THE SEASONALITY OF REPRODUCTION, BODY COMPOSITION, AND ENERGY EXPENDITURE IN NORTHERN RED-BACKED VOLES (*MYODES RUTILUS*)

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THE SEASONALITY OF REPRODUCTION, BODY COMPOSITION, AND ENERGY EXPENDITURE IN NORTHERN RED-BACKED VOLES (*MYODES RUTILUS*)

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Abstract

Arvicoline rodents (voles and lemmings) inhabit high-latitude environments and undergo pronounced seasonal changes in their physiology and behavior. They are an important prey resource in circumpolar regions, and their population numbers can affect the survival and reproductive fitness of many predator and secondary prey species. I studied the effects of seasonality and environmental factors on reproduction and energy allocation in the northern red-backed vole (*Myodes rutilus*), an arvicoline rodent in Alaska known to have bred in winter. My overall aim was to measure the effects of season and environmental factors on the reproductive axis, body composition, and energy expenditure of this animal. I validated a dual-energy X-ray absorptiometry (DXA) apparatus for use in determining fat and lean tissue, body water, protein, and mineral content in *M. rutilus* (R^2 = 0.65 to 0.98, $p < 0.001$ for all parameters). Absolute fat, but not percentage fat changed seasonally. Reproductive organ masses reached peak levels in spring (females) and early summer (males), and significant co-variates were photoperiod, temperature, snow cover, body mass, and percent fat (depending on breeding period and gender). I found one instance of late-summer male non-responsiveness, but no winter breeding. However, 28.2% of captive, lab-raised male voles were non-responsive to short days (*ad lib.* food and water at 20°C), which was within the 20-40% frequency range known for lower latitude species. Differences were found at the gonadal level and pituitary level (testosterone and luteinizing hormone (LH) either varied by group and/or were correlated with testis mass), while differences at the hypothalamic level (gonadotropin-releasing hormone immunoreactivity (GnRH-ir) and gonadotropin-inhibiting hormone (GnIH)-ir cell counts) were inconclusive. Body composition and relative visceral organ mass changed seasonally, and significant covariates were photoperiod (mass, %protein, %mineral), gender (intestines), and temperature (heart). Field metabolic rate did not differ by breeding period, but

was significantly correlated with temperature. Bone mineral density (BMD) of voles was highest in early summer and lowest in winter, whereas the BMD of two hibernating mammals did not change during winter torpor. These findings could help to identify the mechanisms underpinning arvicoline rodent population cycling and to predict physiological and ecological responses of small mammals to different climate change scenarios.

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Chapter 1:

Introduction

1.1 Preface

Arvicoline rodents (voles and lemmings, subfamily *Arvicolinae*) are small, herbivorous mammals abundant in temperate, northern alpine, and arctic ecosystems. They have an effect upon plant communities and soil nutrient loads (Schultz, 1964; Howe and Brown, 1999; Howe *et al.*, 2002; Berg, 2003) and are the major food source for many predatory birds and mammals (Maher, 1970; Wilson and Bromley, 2001; Gilg *et al.*, 2003; Hudson and Bjornstad, 2003). Population cycling is pronounced in arvicolines, and numbers can vary by as many as two orders of magnitude between years (Erlinge *et al.*, 2000; Gilg, 2002; Turchin *et al.*, 2000, Gilg *et al.*, 2003; Framstad *et al.*, 1993). Changes in population numbers of voles and lemmings have a significant effect upon the reproductive success and overall survival of both their predators and alternate prey species (Gilg *et al.*, 2003; Maher, 1970; Wilson and Bromley, 2001; Sittler *et al.*, 2000).

Small rodents inhabit the subnivean space and can survive winter without using torpor or hibernation. Their breeding is typically seasonal, although winter breeding is known to occur in almost every species studied (arvicolines reviewed in Hansson, 1984; Millar, 2001; see also Khlebnikov, 1970; Nelson, 1987; Bronson and Heideman, 1994; Gockel and Ruf, 2001), even at very high latitudes (Whitney, 1976; Hansson, 1984; Kaikusalo and Tast, 1984; Millar, 2001). Successful winter breeding can impact next year's populations, causing increases in overall numbers in summer and later into the season (Khlebnikov, 1970). The success of both seasonal and out-of-season breeding are, therefore, probably linked to the mechanisms underpinning population cycling within arvicoline rodent species (Chitty, 1996).

Some individuals possess the capability to overcome the reproductive challenges and constraints associated with winter to successfully breed out of season and do not respond to a shortening of photoperiod by becoming reproductively inactive. These 'non-responsive morphs' are unique individuals within small rodent populations that possess the genetic capability to maintain the size and function of their reproductive structures under short day lengths (Hansson, 1984; Nelson, 1987). Photoperiod non-responsiveness has not been examined closely in arctic or subarctic species, but in captive colonies of small rodents native to temperate regions 20-40% of the individuals housed under laboratory conditions are non-responsive (Nelson, 1987; Kriegsfeld *et al.*, 2000). Winter breeding in high-latitude populations is rare and indicates that resource availability under the snow is usually insufficient to support both thermoregulatory and reproductive costs in winter (Martell and Fuller, 1979). Many of the details of winter breeding are poorly understood, and the mechanisms arvicolines use to survive and reproduce in high latitudes in the face of reduced forage quality, increased relative metabolic and thermoregulatory demands, and short photoperiods have not been well studied.

Reproduction is also energetically expensive and demands sufficient energy inputs. Yet, many arvicoline rodents do not cache food or store high levels of body fat in the wild (Batzli and Esseks, 1992; Zuercher *et al.*, 1999). Instead, they depend on available resources in the environment year-round to fuel reproduction and maintain normal metabolism. The reproductive condition of rodents is influenced by food availability (Cengel *et al.*, 1978; Hasbrouck *et al.*, 1986; Bronson, 1989), but may or may not be linked to body condition (Cengel *et al.*, 1978; Batzli and Esseks, 1992; Zuercher *et al.*, 1999). Thus, the environmental variables comprising seasonality in high-latitude regions may have important effects upon reproductive and body condition in both seasonal and out-of season breeders. The mechanisms regulating photoperiod non-responsiveness and the influences and interactions affecting reproductive condition and timing, body

condition, energy expenditure, and environmental conditions are poorly understood. However, such details are necessary for understanding how a shift towards a changing or less predictable climate while remaining under a predictable, stationary photoperiod could influence the breeding success and viability of arvicoline rodent populations.

1.2 Overview of Key Facts

1.2.1 Photoperiodism and Seasonal Reproduction

Photoperiodism is the ability of an individual to respond to an exogenous change in daylength through changes in its physiology (*i.e.*, reproductive state) or behavior (*i.e.*, activity pattern). Small mammals in seasonal environments are affected in a number of ways by changes in photoperiod, but most notably in their reproductive condition. Most endotherms in tropical environments respond to non-photoperiodic proximate factors (*e.g.*, temperature, rainfall, humidity, or secondary compounds in green vegetation) rather than photoperiod. Current theories on the existence of photoperiodism in mammals inhabiting seasonal environments are that the trait arose during northern and southern movements from equatorial regions. One suggestion is that mammalian reproduction was coupled with light levels although the cue was not itself a required substrate for reproduction (Austin and Short, 1985; Bronson, 1989). In general, animals outside of equatorial regions (above 30°N or below 30°S) that use photoperiod as a predictor for reproductive activity are thought to have a selective advantage over those that breed opportunistically because they can time peak breeding to coincide with time of peak food availability, beginning the relatively lengthy transition into a reproductive state before new food becomes available (Bronson, 1989).

Photoperiodic information is received at the eye and is passed from the retina along the retinohypothalamic tract (RHT) where it reaches the superschiasmatic nuclei (SCN), the mammalian master circadian clock in charge of regulating activity and physiological patterns (Nelson, 2000). The photic signal is then

passed to the paraventricular nucleus (PVN), the dorsomedial hypothalamus (DMH), the medial forebrain bundle (MFB), and on to the superior cervical ganglion (SCG). The signal is transmitted along post-ganglionic noradrenergic fibers, which extend back into the brain and innervate the pineal gland (Klein *et al.*, 1983; Austin and Short, 1985; Nelson, 2000). Short days (and longer nights) typically act on the pineal glad to produce higher melatonin levels, while long days achieve the reverse (Nelson, 2000). Several hormones affected by melatonin are, therefore, also affected by daylength. The suite of melatonin-stimulated hormones downstream from the pineal gland includes those responsible for reproduction, and upregulation of the reproductive axis can be triggered by an increase in daylength (Wallen and Schneider, 2000).

1.2.2 The Reproductive Axis in Seasonally Breeding Rodents

In all mammals, reproduction is regulated through a complex array of biochemical signals along the hypothalamic-pituitary-gonad (H-P-G) axis. In general, melatonin and other factors bind to receptors on hypothalamic neurons producing gonadotropin releasing hormone (GnRH) and gonadotropin inhibiting hormone (GnIH) in the pre-optic area (POA) and dorsomedial hypothalamus (DMH), respectively. Both GnRH and GnIH cell bodies contain melatonin receptors (Mel_{1c}, and others), which bind to melatonin and induce the transcription and production of neuropeptides (Roy and Belsham, 2002; Ubuka *et al.*, 2005). These two different neurons, although containing similar receptors for melatonin and sex steroids, perform opposite functions in that one stimulates reproduction (via GnRH) and the other inhibits it (via GnIH) (Roy and Belsham, 2002; Tsutsui, 2005).

Only recently, GnIH was identified in the hypothalamus and was found to inhibit gonadotropin release in birds (Tsutsui *et al.*, 2006) and mammals (Kriegsfeld *et al.*, 2006). It is also thought to inhibit the production of GnRH in the hypothalamus (Tsutsui, 2005; Bentley *et al.*, 2006; Tsutsui *et al.*, 2006). Thus,

GnIH may be considered to be both upstream and downstream of GnRH in mammals, as it appears to inhibit GnRH production and/or release at the hypothalamic level and gonadotropin synthesis and release at the pituitary level (Bentley *et al.*, 2006). GnIH is inhibitory upon the synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Bentley *et al.*, 2006). During long days when reproduction is not inhibited, GnRH is upregulated, which stimulates the release of the gonadotropins in the pituitary. This subsequently activates gonadal steroid production and gametogenesis (Austin and Short, 1985).

Mammalian gonadal reproductive anatomy and physiology have been reviewed by Holstein *et al.* (2003). When reproduction is upregulated through increased daylength or food availability, male gonads increase in dry tissue mass of seminiferous tubules, water mass, and gamete density (Sealander, 1967; Zuercher *et al.*, 1999; Johnson and Everitt, 2000). Recrudescence in spring also includes migration of the testes down the lingual canal to a position outside of the body cavity in the developing scrotal sac.

Female voles are thought to be induced ovulators and require either an olfactory signal or copulation to release ova (Clulow and Mallory, 1970; Breed, 1972; Odberg, 1984). However, photoperiod is thought to play an important role prior to this by initializing reproductive readiness (Meek and Lee, 1994). Female voles undergo 3-4 weeks of preparation to produce mature ova after overwintering (Bronson, 1989). Ovaries increase in size due to gonadotropins activating receptors on granulosa cells, which then produce estrogens. The uterus of females also increases rapidly in size during the breeding season due to increased progesterone production, and its lining is made suitable for transportation of male gametes. The corpus lutea and placenta are involved in endocrinological upregulation of female reproduction (Johnson and Everitt, 2000), while the oocyte and growth factors are involved in regulation of ovulation and ovulation rate (McNatty *et al.*, 2004; Fabre *et al.*, 2006).

Negative feedback is an important component of both the male and female reproductive axes. Each level of both axes generates feedback to inhibit all prior points of stimulation, and sometimes undergoes self-inhibition (Bronson, 1989; Johnson and Everitt, 2000; Nelson, 2000; Kriegsfeld *et al.*, 2006). In males, for instance, increases in testosterone (and inhibin) from the gonads generates negative feedback on both the production of LH and FSH at the pituitary, and on GnRH production in the hypothalamus. Production of LH and FSH also generates negative feedback on the hypothalamus to regulate GnRH production and on the pituitary to self-regulate these hormones. Finally, elevated GnRH levels feedback to self-regulate at the hypothalamic level and inhibit pulsing. Feedback pathways are similar along the female H-P-G axis, with estrogen receptors, rather than androgen receptors, on hypothalamic neurons. GnIH neurons also contain receptors for sex steroids (Kriegsfeld *et al.*, 2006).

Downregulation of the H-P-G axis also begins at the eye in photoperiod-responsive animals when decreasing photoperiod translates into higher melatonin levels. This melatonin increase downregulates GnRH and upregulates GnIH, which ultimately leads to a reduction in gonadotropins and sex steroid levels. Males undergo testicular regression in response to decreased gonadotropin levels (Johnson and Everitt, 2000), and testes do not produce sperm in the absence of FSH or testosterone in the absence of LH (Holstein *et al.*, 2003). Testes regress, and any sperm stored in seminiferous tubules and epididymides is either resorped or released in remaining late summer ejaculate. However, most species in the world (living near the tropics) are not primarily photoperiodic. Rather, their seasonal breeding is driven by environmental conditions and food availability in those regions. Animals are well situated to deal with unpredictable events (*e.g.*, drought), which can cause tropical animals to shut down only a part of their reproductive axis while maintaining other parts. This allows them to wait in a state of near-readiness for the opportune time to regain full breeding function (Beebe *et al.*, 2005; Perfito *et al.*, 2006). A single rainfall event can then trigger

and complete the upregulation of the missing portion(s) of the axis within a single day (Bronson, 1989). For instance, pro-GnRH mRNA might continue to be synthesized in the brain while the release of GnRH neuropeptide might be inhibited until a triggering event.

In females, a similar seasonal regression of the reproductive tract also conveys the importance of the breeding window. Downregulation of the H-P-G axis through increased melatonin, negative feedback, and/or other factors, ultimately leads to lower levels of gonadal sex steroids and lower ovary mass. Eventually, no more ovarian follicles develop. Uterine mass also regresses due to decreased levels of progesterone and termination of estrus (although photoperiod-responsive females that are lactating must keep prolactin secretion elevated in the pituitary in order to successfully wean young, even into autumn).

1.2.3 Reproductive Timing in Seasonally Breeding Rodents

In general, seasonal reproduction is regulated through one of two major pathways, by food and energy availability (the central effector pathway) or by stress (the hypothalamic-pituitary-adrenal, or H-P-A axis) (Bronson, 1989). The central effector pathway appears to be the primary regulator of seasonal breeders, which use external environmental factors (usually photoperiod) as cues to couple reproduction to peak energy availability. For animals breeding in high latitude regions, this has important fitness consequences because of the time required to produce functional and viable gametes. Ultimate factors are recognized as those related to the predictability of food availability or food quality. Photoperiod, a proximate factor, is the most stable and reliable predictor of food availability for seasonal breeders, while non-photoperiod proximate factors fine-tune the timing of reproduction, and may be more variable. Photoperiod, therefore, typically acts as a coarse chronological template to coordinate the onset and cessation of breeding, while temperature, rainfall, snow cover, humidity, or other factors play

a relatively smaller, yet still important role, in helping to adjust the breeding window (Austin and Short, 1985).

In arvicoline rodents, the highest energetic costs of reproduction are late gestation and lactation, which can double or triple energy requirements (Kacmarski, 1966; Migula, 1969; Millar, 1977; Innes and Millar, 1981). The cost is usually higher for females, but can also be increased in males. Because it can require up to five weeks for male arvicolines to produce mature spermatozoa after overwintering, and because several weeks are required for females to become capable of releasing mature ova (Austin and Short, 1985; Bronson, 1989) the use of photoperiodic cues in seasonal environments keeps them from wasting several weeks or months priming and synthesizing gametes while vegetation is already available to fuel reproduction (Bronson, 1989). Thus, the selective advantage of responsiveness to an increasing photoperiodic cue (greater than 12.5h of daylight per 24h period) allows them the opportunity to begin reproductive maturation several weeks prior to the emergence of spring vegetation, which will ultimately allow them to time energetically expensive reproductive processes to coincide with times of peak food availability.

Timing of reproduction is important at high-latitudes because voles at the end of winter are usually in their poorest condition, are likely to have few energy reserves, and are unlikely to have access to high quality food. The survival of their offspring depends heavily on coordinating reproductive effort with the correct photoperiod. Two weeks too early or two weeks too late, and fitness is severely diminished (Austin and Short, 1985). This effect becomes more and more pronounced at higher latitudes with more highly seasonal environments where the breeding season is shorter (Bronson, 1989). Yet, at any latitude outside of the tropics photoperiod is the most reliable seasonal predictive cue available to a mammal and is the basis for distinguishing between ultimate and proximate causes of seasonal breeding.

One additional factor that could be related to seasonal reproduction in voles is circadian control. The endogenous circadian clock of mammals is influenced genetically by *Per* and *CLOCK* genes, among others. Light signals are transmitted to the SCN and the pineal gland to regulate circadian rhythms and other important seasonal functions, and circadian rhythms are linked to reproductive state in some, but not all, species of rodents. For example, when exposed to constant light or constant dark conditions in the field and the lab, a large portion of northern red-backed voles (*Myodes rutilus*) become non-circadian, the majority of which ultimately become ultradian or arrhythmic (Stebbins, 1974; Tavernier *et al.*, 2004). Although not studied directly, these results suggest that decreased robustness in circadian control of *M. rutilus* may ultimately be linked to reproductive condition and photoperiod responsiveness.

1.2.4 Photoperiod Non-responsiveness

The separation between the breeding and overwintering seasons within small mammal populations in seasonal environments has been characterized as an energetic bottleneck, as both thermoregulation and reproduction are energetically taxing (Levins, 1968; Nelson *et al.*, 1998). The cessation of breeding (*i.e.*, downregulation of the H-P-G axis and regression of reproductive structures) during winter occurs, presumably, because the costs usually outweigh the reproductive and survival benefits. However, the apparent incompatibility between summer reproduction and winter thermoregulation can be resolved, as almost every arvicoline rodent species studied is known to have bred in winter (reviewed in Hansson, 1984; Millar, 2001). Although high-latitude environments are relatively predictable, populations of voles and lemmings maintain reproductive mechanisms associated with unpredictability through individuals that do not necessarily respond to photoperiod. This provides some reproductive elasticity and may enable winter breeding if environmental conditions are suitable. The non-responsive morph has the opportunity to increase reproductive

output when food or temperature conditions permit. However, this hypothetical increase in reproductive fitness for non-responsive individuals will accrue only if the costs of bearing offspring in winter do not compromise survival of the parent and/or offspring. There may, in fact, be some correlation between the number of litters produced and chance of female survival, but this remains undetermined. Arvicolines that do manage to breed successfully in winter may increase their reproductive fitness over that of their non-breeding conspecifics. The fact that winter breeding exists (although infrequently) in high-latitude vole and lemming populations (Hansson, 1984; Millar, 2001; Whitney, 1976; Kaikusalo and Tast, 1984) suggests that the non-responsive morph has not been eliminated from arctic/subarctic gene pools. However, whether selection for highly seasonal breeding has reduced the frequency of this morph at high-latitudes where successful winter-breeding is not frequent, and therefore may be selected against, is not known.

The mechanisms underpinning non-responsiveness, and the H-P-G axis of different photoperiodic morphs have not been well characterized. However, some evidence suggests that non-responsiveness in arvicolines is regulated at the hypothalamic level in lower-latitude voles (Kriegsfeld *et al.*, 2000). *Peromyscus* non-responsive morphs, for example, do not vary from responsve morphs in their pineal melatonin content, melatonin secretion patterns, brain melatonin receptor numbers, or melatonin receptor binding affinity from that of non-responsive morphs (Blank *et al.*, 1988; Carlson *et al.*, 1989; Weaver *et al.*, 1990; Heideman and Bronson, 1992). Assuming that this is also true of arvicoline rodents, non-responsiveness is likely to be regulated downstream from pineal melatonin and melatonin receptors. The mechanism driving non-responsiveness would, therefore, be related to intraspecific differences in genetic regulation of one or more of GnRH/GnIH, LH/FSH, or testosterone/estrogen synthesis, secretion, and/or receptor density.

Likely energetic and reproductive advantages and disadvantages for individual males are associated with reproductive phenotype. The advantage of non-responsiveness is the potential to increase reproductive fitness. If body size and reproductive phenotype are linked, this might positively influence surface area: volume ratio, which would theoretically increase survivorship to facilitate increased fitness. However, arvicoline species are thought to have increased survival by reducing winter body mass, thereby lowering absolute energy requirements for maintenance costs, and also reducing sex steroid levels to tolerate communal nesting and shared thermoregulatory costs through huddling (Madison *et al.*, 1984; West, 1977; Wolff and Lidicker, 1981; Bronson, 1989). Non-regressed voles with higher sex steroid levels would presumably not huddle with other animals and lose that opportunity to conserve energy.

A well-known immunologic consequence is also associated with reproduction (Nelson and Demas, 1997; Nelson *et al.*, 1998). Short days without cold temperatures boost the immune system, while interaction with even slightly cold temperatures downregulates the system and inhibits responses to noxious stimuli (Nelson and Demas, 1997). Thus, the reproductive axis and the immune system compete for energy reserves in fall and winter, not to mention higher maintenance metabolism as breeders are typically in better condition. Given that most members of vole populations inhibit breeding in late summer, the assumption that deleterious consequences are usually associated with continual reproductive activity is reasonable. If winter breeding had no deleterious consequences, all voles would retain reproductive function in winter.

It is possible that older voles, which might otherwise die in a coming winter, continue extending reproduction and continue passing on their genes for longer periods of time. There could, therefore, be positive fitness consequences for a relatively older animal that maintains its reproductive condition and continues to produce litters into late summer, fall, and early winter, rather than overwintering. Winter breeding is generally thought to occur by an extension of the late-summer

breeding season. However, at least two confirmed instances of spontaneous mid-winter recrudescence have been reported (Kaikusalo and Tast, 1984; Whitney, 1976). Such occurrences may be explained by changes in some acute environmental or nutritional factors (*e.g.*, warmer temperatures or increased food availability) or by genetic variability that has been maintained in the population through natural selection.

1.2.5 Seasonal Breeding and Winter Survival in Alaska

Small nonhibernating mammals in Alaska face a challenging and highly seasonal environment. In winter, ambient temperatures can regularly reach below -40°C. Short photoperiods, high winds, and accumulation of snow and ice make life challenging, and the presence of a snow pack is important for insulation, especially in non-coastal regions that do not benefit from the milder climate associated with the ocean. In summer, interior Alaska's temperatures can exceed 35°C. Despite this, nonhibernating rodents like voles and lemmings maintain relatively constant year-round body temperatures, heart rates, and metabolic rates. They undergo a set of physiological and behavioral changes and forage continually under the snow.

In winter, activity is severely reduced in arvicolines, and animals form communal nests to decrease conduction with the ground and convection with the air within the subnivean space (West, 1977). The skin and pelage undergo noticeable seasonal changes (Sealander, 1972). The skin is much thicker and hair is much longer in winter, reducing the effects of heat dissipation and heat loss by convection and conduction. Color and reflectance of pelage also change seasonally. Body mass is often reduced, presumably to minimize absolute energy requirements while maintaining an active metabolism (Whitney, 1976; Zuercher *et al.*, 1999). Voles undergo metabolic adjustments, including winter increases in their field metabolic rate (Holleman *et al.*, 1982), maximum metabolic rate (Rosenmann *et al.*, 1975), capacity to synthesize catecholamines, and sensitivity

to norepinephrine (Fiest and Rosenmann, 1976), all of which are likely to benefit their cardiovascular and metabolic response to cold. Some rodents, such as Siberian hamsters (*Phodopus sungorus*), upregulate elements of non-shivering thermogenesis under short day lengths through increases in gene expression in brown adipose tissue (BAT) (Demas *et al.*, 2002). In these experiments, short days decreased white adipose tissue (WAT) mass and leptin mRNA, increased WAT β_3-adrenoceptor mRNA, and induced retroperitoneal WAT uncoupling protein-1 mRNA while increasing BAT uncoupling protein-1 and peroxisome proliferator-activated receptor-γ coactivator-1 mRNAs. This short day-induced decrease in adiposity of these rodents could be due to a coordinated suite of WAT and BAT gene transcription changes, ultimately increasing lipid mobilization and utilization. During breeding in arvicoline rodents, however, thermogenic capacity can decrease via downregulation of cytochrome c oxidase (COX) and uncoupling protein-1 (UCP-1) in BAT (Li and Wang, 2005). This presumably helps to conserve energy for spring and summer reproduction at thermalneutral temperatures.

Trends in body condition of Alaskan arvicolines in winter have been generally characterized as having increases in absolute mass of BAT and decreases in white adipose tissue (WAT) and protein content (Sealander, 1972; Zuercher *et al.*, 1999). Although small rodents differ widely in seasonal fat deposition trends across species and locations, field studies in Alaska describe fat percentages as generally low and only slightly higher in summer and winter than in spring and fall (Sealander, 1972; Zuercher *et al.*, 1999). Arvicoline rodents can select a wide variety of different foods from hypogeous and other fungi to monocots to berries (Bangs, 1984), and they are likely to undergo significant seasonal changes in diet. Fungi or berries, which are most readily available in late summer and fall, are often a better energy source than other food items. Such differences may affect the condition of voles, including body mass, size of liver (where glycogen is stored), protein stores, size of testes, and/or and gamete production. The adrenal

organ mass of nonhibernating voles also shows a significant seasonal change that is positively correlated with photoperiod (Sealander, 1967). Other non-reproductive visceral organs are likely to change seasonally. This could convey some importance about the seasonal performance, function, or importance of different components (Zuercher *et al.*, 1999).

1.3 Aims and Objectives of Experimental Chapters

1.3.1 Determinination of Body Composition Using DXA

Body composition is important to reproduction and can change seasonally. The composition of small mammals can be determined non-destructively using dual-energy X-ray absorptiometry (DXA) (Pietrobelli, 1996), and DXA apparati have been validated for precision and accuracy in different species (Nagy and Clair, 2000; Brommage, 2003; Johnston *et al.*, 2005). The aim of Chapter 2 is to validate a DXA apparatus for non-destructive use in small, free-living and lab-raised northern red-backed voles. I measured and tested body composition often throughout my experiments, and I derived predictive algorithms that can accurately and non-destructively measure fat mass, lean mass, total body water, protein content, mineral content, and fat-free mass in this species.

1.3.2 Seasonality of Reproduction in Responsive and Non-responsive Voles

The aim of Chapter 3 is to determine the timing of reproduction, the pattern of organ mass change, the factors affecting timing and seasonal change, and the frequency of photoperiod non-responsiveness in the northern red-backed vole, *Myodes rutilus* in the field and the laboratory. Reproduction is typically seasonal in arvicoline rodents, but out-of-season breeding has been documented in most species, including *M. rutilus* (reviewed in Hansson, 1984; Millar, 2001). Reproductive condition may be related to environmental parameters or body condition in breeding and non-breeding seasons. Additionally, photoperiod responsive and non-responsive phenotypes may be expressed at higher

frequencies under favorable laboratory conditions than in field populations (Kriegsfeld *et al.*, 2000). However, it is not known whether such frequencies of non-responders in high-latitude arvicoline rodents differ from those at lower latitudes.

1.3.3 Differential Regulation of the H-P-G Axis

The aim of Chapter 4 is to determine where differences existed along the H-P-G axis between responsive and non-responsive voles. Non-responsive and responsive male *M. rutilus* were classified by phenotype under short daylength (*i.e.*, testis mass relative to long day animals). This inherent difference in photoperiod responsivity may be mediated at any point along the H-P-G axis. I mapped GnRH and GnIH neurons in the hypothalamus of voles, and I measured differences in hypothalamic, pituitary, and gonadal hormones in different experimental groups. I also tested for differences in age and body mass within each group and whether splenic mass (a general indicator of immune function) is related to photoperiod or reproductive phenotype.

1.3.4 Seasonality of Composition, Internal Organ Mass, and Metabolic Rate in Voles

The aim of Chapter 5 is to determine whether seasons and environmental factors were related to any of six elements of body composition, any of eight relative visceral internal organ masses, or field metabolic rate (FMR) in northern red-backed voles (*Myodes rutilus*). Small mammals in seasonal environments may allocate mass and energy to certain tissues, organs, and processes over others in specific seasons. This may reflect a temporal level of importance for a given parameter or structure. In high-latitude environments, seasonality is likely to have an impact upon the body composition, relative internal organ masses, and field metabolic rate (FMR) of a free-living vole. Strong relationships could exist between environmental variables and physiological structures and functions that

would convey information about how or why a vole allows changes in its physiology in response to season to facilitate breeding or winter survival.

1.3.5 Seasonal Changes in Bone Mineral Density of Voles

The aim of Chapter 6 is to test whether the bone mineral density (BMD) of a high-latitude species, the northern red-backed vole, changed seasonally, and if so, whether these changes were correlated with differences in sex, body morphology, or photoperiod (a mediator of activity and concentrations of reproductive hormones in high-latitude voles and lemmings). The minimum, maximum, and seasonal change in BMD of voles could be affected by changes in their behavior and physiology, including body mass, activity, reproductive hormones, and other hormones.

1.3.6 Seasonal Changes in Bone Mineral Density of Hibernators

The aim of chapter 7 is to determine the effect of hibernation duration and ambient temperature on the body mass and bone density of two mammalian hibernators, the arctic ground squirrel (*Spermophilus parryii*) and the American black bear (*Ursus americanus*). Osteopenia occurs in the northern red-backed vole (*M. rutilus*), a non-hibernator, from late summer to late winter (Stevenson *et al.*, 2009). The potential for a similar decrease in bone density during this time also exists for hibernators, despite reductions in blood flow, metabolic rate, and body temperature that could disrupt bone resorption while in torpor. This work will provide a frame of reference for comparisons with non-hibernators overwintering in Alaska.

1.4 Conclusion

Elements of seasonality are likely to be strongly tied to elements of reproduction, body composition, and energy expenditure in the northern red-backed vole, a high-latitude rodent. By measuring intraspecific variation in

reproductive responsivity in field and laboratory populations, mechanisms of differential regulation of the H-P-G axis, and determining environmental and body composition correlates, I made it possible to understand how this animal responds to changes in season and climate. These studies also provide insight into which factors are likely to be responsible for driving inter- and intra-annual differences in breeding and population cycling.

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Chapter 2:
Dual-Energy X-Ray Absorptiometry (DXA) Can Accurately and Nondestructively Measure the Body Composition of Small, Free-Living Rodents[1]

2.1 Abstract

Dual-energy X-ray absorptiometry (DXA) is a non-destructive technique that can potentially measure specific components of whole body composition in free-living and lab-raised animals. Our aim was to test the ability of DXA to measure the composition of a common arvicoline rodent, the northern redbacked vole (*Myodes rutilus*). We used a DXA apparatus to obtain measurements of fat mass (FM), lean mass (LM), bone mineral content, bone mineral density, and fat-free mass (FFM) in free-living and lab-raised vole carcasses. We then used chemical carcass analysis to derive predictive algorithms for actual values of FM, total body water (TBW), total protein (TP), total mineral (TM), LM, and FFM. Unexplained error in the equations for all voles grouped collectively ranged from $R^2 = 0.82$ to $R^2 = 0.98$. The DXA FM measurement had the highest coefficient of variation, and it was higher for free-living voles than for lab-raised voles. However, FM can be determined by difference with excellent precision using the FFM equation ($R^2 = 0.98$). We also derived corrective terms for passive integrated transponder (PIT) tagged animals. Thus, DXA is a non-lethal, non-destructive tool capable of precisely and accurately measuring many specific parameters of whole body composition in small, free-living and lab-raised rodents.

2.2 Introduction

Measurements of body composition are important because they characterize an animal's biological make-up and may indicate nutritional state or life-history stage. Composition may include the type and percentage of an animal's fuel

[1]Stevenson KT, van Tets IG. 2008. Dual-Energy X-Ray Absorptiometry (DXA) can accurately and non-destructively measure the body condition of small, free-living rodents. *Physiol Biochem Zool* 81(3):371-382.

reserves, the hydration state of an animal, or other measurements that describe portions of the animal's total chemical make-up. The body condition of an animal has traditionally referred to some measure of its fitness, either in a broad sense (*e.g.*, measures of reproductive condition) or in a more specific sense (*e.g.*, body fat percentage). Although these terms have, at times, been used interchangeably, I emphasize the whole biological composition in this study rather than the fitness or condition of an animal.

The body composition of rodents has been a largely important factor for clinical and dietary trials in the laboratory, but it is also important for studying the physiological ecology of small herbivores in varying environments. Composition has previously been measured using an assortment of methods, but each has been problematic for small rodents. Body-mass indices are generated by calculating a ratio of length to mass, but can be non-repeatable and inaccurate reflections of actual composition (Krebs and Singleton, 1993; Schulte-Hostedde *et al.*, 2001a). The negative correlation between body water and the amount of fat that some animals carry has also been used to estimate condition (Winstanley *et al.*, 1998), but these relationships are not always consistent in small mammals (Schulte-Hostedde *et al.*, 2001a). Total body electrical conductivity (TOBEC) instruments have been used to measure the differences in electrical properties of lean tissue and body fat and can theoretically predict three body composition components: body water content, lean mass, and fat mass. However, these devices vary heavily in their precision and accuracy, and thus, their actual ability to measure the different parameters of body composition (Walsberg, 1988; Castro *et al.*, 1990; Zuercher *et al.*, 1997; Unangst and Wunder, 2001). For instance, Zuercher *et al.* (1997) noted that two different TOBEC instruments produced poor fat estimates from lean mass predictions, and neither could be used to accurately measure changes in total body fat of individual voles. Additionally, TOBEC instruments require that animals be maintained at normal levels of hydration and body temperature (Waslberg, 1988).

Chemical carcass analysis or "proximate analysis" is accurate but lethal (Batzli and Pitelka, 1971; Zuercher *et al.*, 1997; Batzli and Esseks, 1992; Schulte-Hostedde *et al.*, 2001a; Schulte-Hostedde *et al.*, 2001b; Mata *et al.*, 2006). It destroys the assessed tissues, preventing any further analyses, and is difficult to justify for research on threatened or endangered species. However, technological advances in radiology and physiology have led to the formulation of a non-destructive, non-invasive method for determining body composition known as dual energy X-ray absorptiometry (DXA). DXA uses two X-rays, one of a high energy dose (70-90 keV) and one of a low energy dose (15-40 keV), to measure body composition. As photons emitted by the machine traverse a subject's tissues, physical interactions take place that reduce a beam's intensity (attenuation). These photons are either absorbed or scattered by interactions of Compton scattering and photoelectric effect (Pietrobelli *et al.*, 1996). The differential attenuation of these X-rays through bone, lean tissue, and fat are quantified by the DXA apparatus and provide a unique system of measurement. When photons at two different energies pass through an absorber, the beam attenuation at the lower energy can be expressed as a ratio (R) of the beam attenuation at the higher energy.

Every atomic element has a specific R value and a characteristic mass attenuation coefficient (μ) at high and low energies (Pietrobelli *et al.*, 1996). A sigmoidal association between these R-values and their corresponding atomic numbers exists, with the main constituents of water and organic compounds (H,C,N,O) having small R-values, soft tissue minerals (Na, K, Cl) having large R-values, and elements primarily in bone mineral (Ca) having even larger R-values (see Piterobelli *et al.*, 1996). These R-values are unique and range from 1.2058 to 1.2289 for triglycerides and fatty-acids, and from 1.2906 to 2.9939 for many lean components (*e.g.,* protein, glycogen, extracellular and intracellular water, soft tissue minerals) and bone mineral. The DXA method for estimating the three major components of composition (fat mass, lean mass, and bone) is to separate

pixels into those with soft tissue only (fat + lean) and those containing soft tissue + bone mineral. Pixel separation, or "point typing", is then accomplished by computing an R-value for each pixel in a total body scan, and an R-value threshold is set to distinguish between pixels that include bone mineral and those that consist of only soft tissue (Pietrobelli *et al.*, 1996). Thus, the R-values obtained by DXA are used to identify the unknown components of body composition.

Initially developed as a tool for clinical research (Peppler and Mazess, 1981; Gotfredsen *et al.*, 1986), DXA apparati have been used to study or develop treatments for osteoporosis (Grodem *et al.*, 1996; Michaelsson *et al.*, 1996), vertebral deformity (Ross *et al.*, 1995), obesity (Hendel *et al.*, 1996; Carey *et al.*, 1996), idiopathic renal stone disease (Trinchieri *et al.*, 1999), and a wide variety of other disorders and conditions in humans and lab mice (reviewed by Albanese *et al.*, 2003). The application of DXA, however, has since broadened to the fields of agriculture, animal science, and veterinary science. It has been used to measure the body composition of sheep (Pouilles *et al.*, 2000), pigs (Mitchell *et. al.*, 1998; Nielson *et al.*, 2004), chickens (Mitchell *et al.*, 1997; Swennen *et al.*, 2004), dogs (Toll *et al.*, 1994), and other domestic and agricultural animals. The success of these studies has led to recent applications of DXA in wildlife physiology. For example, DXA has been used to measure body composition in collared lemmings undergoing photoperiod-induced weight gain (Hunter and Nagy, 2002), the effects of long-term dietary restriction on rhesus monkeys (Blanc *et al.*, 2003), the effects of different diets on fat and lean mass changes in grizzly bears (Felicetti *et al.*, 2003), and the body composition of passerine birds (Korine *et al.*, 2004). Almost all of these studies have used colonized, lab-raised subjects (with some exceptions, *e.g.,* Korine *et al.*, 2004), and captive animals typically have very different body compositions than free-living animals. For instance, free-living brown lemmings (*Lemmus sibiricus*) and other arvicoline rodents often contain only 5% body fat, while lab-raised lemmings may reach

40% body fat (Batzli and Esseks, 1992). In studies where DXA has been used on free-living terrestrial mammals, it appears to have usually been limited to measuring only changes in bone mineral content and density (Hiyaoka *et al.*, 1994; Bjora *et al.*, 2001; Dirrigl *et al.*, 2004; Garriga *et al.*, 2004) with one exception (Lehmer and Van Horne, 2001). This is presumably because DXA's bone measurements are known to have greater internal accuracy than its soft tissue measurements (Nagy and Clair, 2000).

Previously, DXA has been validated by proximate analysis for use on the non-cranial region of lab-raised rodents. Brommage (2003) validated a machine for use with decapitated mice, and Nagy and Clair (2000) used the PIXImus DXA apparatus (Lunar/G.E.) and software to digitally exclude head regions of full-bodied mice. Johnston *et al.* (2005) also excluded cranial and tail regions of obese and wild-type mice, Siberian hamsters, and bank voles to determine capabilities of the PIXImus2 for predicting fat mass. These validations involving lab-raised rodents are likely of most value to biomedical studies that focus on the effects of a given treatment on core body composition only. In physiological ecology and wildlife physiology studies, however, research concerns may lie more in the realm of whole body composition and response to seasonal or environmental variables. For instance, the skull and brain add significant mass to the body, may change seasonally (Yaskin, 1984; Zuercher *et al.*, 1999), and should be included in any morphological measurements relative to body mass.

Unlike proximate analysis, DXA's non-destructive nature allows for the determination of body composition in live, immobilized animals during repeated measures experiments, as well as in lethal-trap studies for which the preservation of internal tissues and organs is desired. Most rodent-sized DXA apparati are, in fact, portable, non-destructive, inexpensive to maintain and operate, and time efficient (~3 minutes per scan on a sedated animal). Thus, a DXA instrument could be an ideal tool for non-destructively measuring the composition of small mammals in a broad array of field and laboratory-based wildlife physiology

studies if it could be validated for whole-bodied, leaner, free-living rodents with a high degree of precision and accuracy. The aim of my study was to use proximate analysis to validate the measurements of a DXA apparatus for use on whole free-living and lab-raised northern redbacked voles (*Myodes rutilus*).

2.3 Methods

Ten free-living northern redbacked voles were collected from Chugach State Park in southcentral Alaska and from forested areas around the University of Alaska Anchorage (UAA) campus and frozen at -20°C. Twelve lab-raised *M. rutilus*, which were visibly fatter than the free-living voles, were donated from the University of Alaska Fairbanks (UAF) captive Bonanza Creek colony (Tavernier *et al.*, 2004). These voles were euthanized, frozen at -20°C, and shipped to the UAA campus on ice. Procedures were approved by the UAA Institutional Animal Care and Use Committee (IACUC) committee (protocol# 2005VanTe1).

2.3.1 DXA Analysis

Voles were individually thawed to room temperature and weighed on a laboratory balance. Fat mass (FM_{DXA}), lean mass (LM_{DXA}), bone mineral content (BMC_{DXA}), and bone mineral density (BMD_{DXA}) were measured using a PIXImus2 DXA apparatus (Lunar/GE corp.) and accompanying Lunar PIXImus2 2.00 software. Quality control statistics of the apparatus were set to allow no more than 0.2% error during calibration with a quality control phantom standard (BMD = 0.0630g/cm^2, %Fat = 9.5%). Voles were individually thawed to room temperature and placed on the scanning platform of the apparatus, dorsal side up. Appendages were stretched out to the corners of the rectangular platform and the tail curled under the hind leg. Larger voles had to be positioned diagonally on the platform so that their entire carcasses could be scanned and analyzed by the machine. All animals were scanned at least five times with repositioning of the carcass between each measurement.

2.3.2 Chemical Carcass Analysis

Following DXA scans, whole vole carcasses were reweighed and subsequently homogenized in beakers using a Kinematica© homogenizer. Homogenate was dried in an oven at 60°C to a constant mass to determine total body water (TBW) content. For three of the voles, sub-samples of homogenate were separated, dried in triplicate, and averaged to ensure precision in the drying technique. The total mineral (TM) content of the dried homogenate was determined by weighing triplicate samples of homogenate after ashing in a muffle furnace at 500°C for over eight hours. Non-bone mineral content (NBMC) was determined by calculating the difference between the TM value and the PIXImus2 DXA bone mineral content value (BMC_{DXA}; NBMC = TM - BMC_{DXA}).

We determined the total protein (TP) content of each vole by measuring the nitrogen content of dried homogenate in triplicate with a carbon, nitrogen, hydrogen (CNH) spectrophotometer (Leco, Inc.) at the UAF Agricultural and Forestry Experimental Research Station (Palmer, Alaska), and multiplying by the standard nitrogen-protein conversion factor of 6.25 for animal protein (Jones, [1931] 1941; Jones *et al.*, 1942; Food and Agriculture Organization/World Health Organization, 1973; Food and Agriculture Organization, 2003).

We used an accelerated solvent extractor (ASE) (Dionex Inc.) with a mixture of 65%-35% chloroform-methanol ($CHCl_3$/MeOH) solvent, and followed the methods of Dodds *et al.* (2004) to chemically determine vole fat mass (FM). Solvent type has been known to influence lipid recovery (Giergielewicz-Mozajska *et al.*, 2001; Dodds *et al.*, 2004), and $CHCl_3$/MeOH was chosen because it is strongly polar and a very effective and consistent extractor of lipid (Dodds *et al.*, 2004). Accelerated solvent extraction is more effective than traditional methods of total body lipid extraction, such as soxhlet, because the solubility of analytes and diffusion rates are increased, strong interactions between analytes and matrix components are weakened or disrupted, and viscosity and surface tension of

solvents are decreased (Giergielewicz-Mozajska *et al.*, 2001). Each of these allows for a more accurate recovery of total lipid.

The extractor injected solvent into heated (100°C) metal cells that contained subsamples of dried homogenate (100-500mg) and hydromatrix material. The extract was filtered into collection vials, mixed with 0.9% NaCl solution, and passed through a sodium sulfate matrix with chloroform rinse. Samples were analyzed in duplicate or triplicate and averaged. Data from one free-living vole and one lab-raised vole were excluded from lipid recovery analysis because I was unable to obtain multiple values for these animals.

Our equipment and techniques were used to measure the total lipid recovery of a standard reference material against its certified National Institute of Standards and Technologies (NIST) value (Dodds *et al.*, 2004). Sample lipid recovery values exceeded the NIST value by 3.3%, and I corrected for this overestimate by multiplying all of my percent lipid recovery values by a factor of 0.967.

The percent lipid recovery from samples of dried, free-living vole homogenate averaged 15.5±1.6% (due to the absence of water), which was within the percent lipid recovery range of between 2% and 20% that had been validated for my equipment and techniques (Dodds *et al.*, 2004). Percent lipid recovery from dried, lab-raised vole homogenate, however, was much higher, reaching up to 53.4±3.6% and well outside the validated range of the extractor.

In the free-living voles, the sum of all chemically analyzed components (TBW + TP + TM + FM) equaled 98.5±0.3 % of the total gravimetric body mass ($Mass_{GRAV}$). The small amount of residual mass (approximately 1.5%) was made up of the non-analyzed components, that is all other dry, fat-free, ash-free, non-protein mass (*e.g.*, glucose, glycogen, sugars, vitamins, DNA, RNA, fiber components, etc.). Although not measured chemically in this study, these components, collectively termed residual lean mass (LM_{RESID}), are measured by

DXA and are included in the DXA lean mass (LM_{DXA}) measurement (Pietrobelli *et al.* 1996).

As the sum of all chemically analyzed components in the lab-raised vole homogenate equaled 103.1±1.4% of gravimetric body mass, I concluded that the lipid recovery technique of Dodds *et al.* (2004) overestimates actual lipid content and introduces greater variation in these heavily concentrated samples. To determine the fat-free mass of these lab-raised animals, I added the average LM_{RESID} value calculated for free-living voles (0.207g) to the sum of each lab-raised vole's fat-free mass components that were chemically analyzed (TBW + TP + TM). The actual average LM_{RESID} value should be quite similar in the two groups because a heavy increase in percent body fat does not constitute a proportional or heavy increase in LM_{RESID}. I was then able to determine each lab-raised vole's actual fat content by calculating the difference between the chemically-derived fat-free mass and the gravimetric body mass. For both free-living and lab-raised voles, fat content was measured by both a DXA scan and by another analytical process. Although the lipid content of the lab-raised vole homogenate could not be determined by direct chemical extraction because it was well outside the validated range for ASE, greater than 99% of the fat-free mass was determined chemically in order to calculate an actual value of fat by difference for lab-raised voles.

I defined the total lean mass (LM) as the sum of the TBW, TP, and residual lean mass components. I do not include bone mass in the LM measurement (although some authors do) because the PIXImus 2.00 software does not include bone mass in its DXA lean mass (LM_{DXA}) measurement. The 'percent fat' value displayed by the PIXImus2 is actually a measure of fat as a percentage of all soft tissue and not as a percentage of total body mass that includes bone. I defined fat-free mass (FFM) as the sum of total LM and bone mineral and defined FFM_{DXA} as the sum of all DXA-derived fat-free mass components displayed in the PIXImus2 output screen ($LM_{DXA} + BMC_{DXA}$).

I also evaluated the effect of passive integrated transponder (PIT) tags on direct DXA measurements. Free-living voles (n = 38) that had been injected with subcutaneous tags (Destron Technologies Inc., TX1440L10S-CD81740) between their scapulae as part of a different study were recaptured in Chugach State Park, Alaska, euthanized with halothane, and frozen at -20°C. I thawed these voles to room temperature and scanned animals on the DXA platform. Tags were then removed by applying physical pressure and forcing them through the skin. Animals were reset on the platform with minimal repositioning and scanned a second time.

I used SPSS statistical software (version 14.0) for data analysis. Regression analysis was used to derive relationships between the body composition parameters measured chemically by proximate analysis and those measured by DXA. Simple linear regressions were used in all but one statistical comparison (FM). A stepwise multiple regression was employed only in my determination of actual FM, and I used FM_{DXA} and LM_{DXA} as independent input variables (Nagy and Clair, 2000). The selected model of best fit, however, used only the FM_{DXA} variable and not the LM variable, so I have also reported this as a simple linear regression. Bland-Altman graphs were used where appropriate to show agreement between established chemical techniques and DXA measurements of the same parameter (Bland and Altman, 1986). To determine the effect of PIT tags on free-living voles, paired t-tests were used to determine the mean differences between DXA values recorded in each of the two scans (tagged vs. untagged).

2.4 Results

I tested the precision of each of the four DXA measurements directly recorded by the PIXImus2 2.00 software (FM_{DXA}, LM_{DXA}, BMC_{DXA}, and BMD_{DXA}) by calculating a coefficient of variation (CV) for each parameter in each animal that was measured by DXA with repositioning of the carcass between scans. The CVs were averaged for three groups: the lean free-living voles, the fatter lab-raised

voles, and all voles grouped collectively (Table 2.1). For all voles grouped collectively, the FM_{DXA} measurement had the highest average CV (6.8%). The LM_{DXA} measurement, which comprises most of the FFM_{DXA} measurement, had the lowest average CV (1.6%). The CV for the BMC_{DXA} measurement was next lowest (2.3%), followed closely by the BMD_{DXA} measurement (3.6%), which the machine calculates by dividing the BMC_{DXA} value (g) by the measured bone area (cm^2) to determine bone density (g/cm^2).

We grouped all voles collectively in each of my regression analyses. To test whether DXA estimates total body mass accurately, I derived a relationship between the gravimetric mass ($Mass_{GRAV}$) and the DXA-derived body mass ($Mass_{DXA}$), that is, the sum of all DXA-derived body composition components displayed in the output screen ($FM_{DXA} + LM_{DXA} + BMC_{DXA}$). The DXA apparatus overestimated body mass slightly, but consistently, and the predictive equation contained virtually no unexplained error (Fig. 2.1).

For all voles grouped collectively, the DXA apparatus predicted the tested components of body composition with an R^2 of 0.82 (TM), 0.88 (TP), 0.98 (TBW, LM, FFM) and 1.00 ($Mass_{GRAV}$) (Figs. 2.1 thru 2.4). DXA values agreed closely with the corresponding chemical values of the same parameter (Figs. 2.3d, 2.4b, and 2.4d). Predictive equations and reported errors for all tested components of body composition are listed in Table 2.2. Separate regression lines and predictive equations were added only to the FM graph to show the difference in results between the free-living and lab-raised voles (Fig. 2.4a) because of an apparent difference in magnitude of average CV and because I was interested in the instrument's ability to directly measure FM specifically in lean, free-living voles.

Finally, standard subcutaneous rodent PIT tags caused a significant ($p<0.05$) increase in each of the four DXA parameters directly recorded by the PIXImus2 2.00 software (Table 2.3), and I derived corrective terms to allow for safe, accurate measurements of sedated animals without the need for tag removal. To acquire an accurate measurement of each parameter in a tagged animal, DXA

values should first be corrected using these terms before using any of the predictive equations.

2.5 Discussion

The results of this study are consistent with a prior validation of the same apparatus on decapitated lab-mice in which CVs were higher in the FM_{DXA} measurement than in the other DXA measurements and in which FM was overestimated by DXA (Nagy & Clair, 2000). Lean mass and total body mass were also slightly overestimated by DXA in this study. In comparing the precision of DXA's measurements between free-living and lab-raised vole groups, the only difference of any noticeable magnitude in average CV was in the FM_{DXA} measurement, with the difference between groups equaling 1.8%. Absolute values of the difference between average CVs in free-living and lab-raised voles were only 0.2%, 0.0%, and 0.3% for LM_{DXA}, BMC_{DXA}, and BMD_{DXA}, respectively (Table 2.1). This suggests that DXA's capability to detect and measure fat levels by the FM_{DXA} measurement alone approaches its lower limit of reliability for very lean, free-living animals. In general, there seems to be some loss in precision in the FM_{DXA} measurement as fat levels diminish. This result is consistent with results of a prior study in which both obese and leaner wild-type rodents scanned on two similar PIXImus2 DXA machines (with head and tail regions excluded) yielded the same FM_{DXA} readings for obese animals, but not for leaner, wild-type animals (Johnston *et al.*, 2005). They concluded that DXA apparati using the same software could use the same correction equation to accurately predict FM for obese mutants, but not for lean, wild-type animals. Additionally, small systematic errors in DXA soft tissue analysis have been known to arise with variation in fluid balance (Pietrobelli *et al.*, 1998), which could potentially affect free-living voles more than lab-raised voles.

In this study, predictive equations for fat-free mass (FFM) contained very little unexplained error [$FFM = 0.97(FFM_{DXA}) + 0.13$; $R^2 = 0.98$; SEE = 0.65]

(Fig. 2.4d). This is largely due to the machine's excellent ability to predict total body water via the lean mass measurement [TBW = 0.76(LM_{DXA}) + 0.12; R^2 = 0.98; SEE = 0.60] (Fig. 2.3a). In general, water comprises approximately 70% of a free-living vole's total body mass, over 75% of its fat-free mass, and almost 80% of its lean mass. Hence, the precision of the LM_{DXA} measurement (Table 2.1) and the accuracy with which it predicts TBW (Fig. 2.3a) are very important contributors to the machine's ability to predict LM and FFM with very little unexplained error (R^2 = 0.98 for both; Figs. 2.3c and 4c, respectively). Percent body fat can, therefore, be predicted by DXA with tremendous accuracy for all voles using the $Mass_{GRAV}$ and FFM_{DXA} values to calculate FM by difference. However, caution must be used to ensure that trapped animals do not dehydrate and are weighed both at the time of both capture and DXA analysis. Drawing blood or removing tissues from an animal will also affect FFM. In such cases, it may be best to use the FM_{DXA} equation because the FM_{DXA} measurement is not dependent upon LM values and retains slightly reduced, yet satisfactory precision and accuracy. In instances where voles are collected from different seasons, body mass and composition are likely to vary. In such instances, the relative body fat levels of pregnant or lactating females, very large individuals, or even very small individuals could resemble the percentage body fat of the leanest lab-raised voles. The FM_{DXA} equation for free-living or all voles could be used if there were high numbers of gestating, lactating, or relatively fatter voles, or if there was high variability in the body composition, season, or latitude at which animals were trapped (Fig. 2.4a). In any instance, the equation selected should be used consistently throughout comparisons of free-living animals.

Lean mass in free-living and lab-raised voles can be predicted with excellent accuracy (R^2 = 0.98) using DXA. Although LM is comprised mostly of water, it is important to keep in mind that TP and LM_{RESID} are also contributing factors. The TP of voles comprises a much smaller portion of the LM, and a slightly more reliable and independent measurement of TP could be obtained by non-lethal

means. By using deuterium dilution to measure the TBW of a scanned animal, and by continuing to assume that changes in LM_{RESID} are negligible, TP could be independently measured using $TP = LM - TBW - LM_{RESID}$ where LM is derived from the equation in Figure 2.3c, TBW is derived from the equation in Figure 2.3a, and LM_{RESID} is constant (0.207g in the case of *M. rutilus*). Estimates of TP using the derived DXA algorithm (Fig. 2.3b) are, therefore, highly reliable under the assumption that %TBW is constant in all study animals and less reliable when not measured independently.

Subcutaneous PIT tags embedded in recaptured animals caused a significant but predictable increase in all parameters reported by the DXA software (Table 2.3). Field studies utilizing portable DXA machines and the same type/size tag should use these correction factors to avoid including the effect of tags in the data and thus elevating the level of actual body composition values in immobilized animals. If other types or sizes of PIT tags are used, a DXA scan before and after tagging could be used to define similar correction terms. The digital cropping function available on the Lunar PIXImus2 DXA apparatus might also be able to exclude both embedded and external tags without affecting measurements.

Unfortunately, the Lunar PIXImus2 DXA machine used in these experiments is no longer being manufactured, and there is at present no portable equivalent available on the market. Therefore a used or remanufactured Lunar machine would have to be purchased for field studies that require a portable machine. Alternatively, animals could be brought to a facility where a stationary DXA machine is available, although the feeding, handling, and transport of the study animals may change their body composition.

The PIXImus2 DXA apparatus can be used to accurately determine values of several body composition parameters in free-living and lab-raised voles. It can provide reliable data while preserving population numbers, even in recapture studies where animals are PIT tagged. It is uncertain whether a DXA apparatus could accurately determine body composition in animals smaller than 10g, such as

shrews, because their bones and fat deposits are very small and may not be detected by DXA. The derived equations in this study should, however, be applicable to all small free-living and lab-raised rodents of 10-35g in weight, encompassing species of *Clethrionomys, Microtus, Mus, Peromyscus,* and others. For these rodents, DXA represents a non-lethal, non-destructive tool that is capable of measuring many specific parameters of whole body composition in small, free-living and lab-raised individuals with excellent precision and accuracy.

2.6 Acknowledgements

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2.8 Tables

Table 2.1 Precision of DXA Measurements. Average percentage coefficients of variation (CVs) are listed for direct DXA measurements of fat mass (FM_{DXA}), lean mass (LM_{DXA}), bone mineral content (BMC_{DXA}), and bone mineral density (BMD_{DXA}) in free-living and lab-raised voles. FM_{DXA} measurements were least precise, having the highest average CVs. The LM_{DXA} and BMC_{DXA} measurements, which together make up FFM_{DXA}, had the lowest average CVs. The BMC_{DXA} measurements were more precise than BMD_{DXA} measurements, which depend on a calculation of bone mass and area (g/cm^2), and are more likely to be affected by animal repositioning. The only notable difference in magnitude of average CV between the two groups of voles was in the FM_{DXA} measurement, which was higher in free-living voles than in lab-raised voles (difference of 1.8%). This suggests that DXA's lower limit of fat detection is being approached in leaner, wild animals.

DXA Measurement	Free-living (n = 10; %)	Lab-raised (n = 12; %)	Difference (%)	All Animals (n = 22; %)
FM_{DXA}	7.8	6.0	1.8	6.8
LM_{DXA}	1.5	1.7	-0.2	1.6
BMC_{DXA}	2.3	2.3	0.0	2.3
BMD_{DXA}	3.4	3.7	-0.3	3.6

Table 2.2 Predictive Algorithms for Body Composition Parameters Based on DXA Measurements. Equations and standard errors of the estimate (SEEs) are listed for actual values of gravimetric body mass ($Mass_{Grav}$), total mineral (TM), total body water (TBW), total protein (TP), lean mass (LM), fat mass (FM), and fat-free mass (FM) in free-living and lab-raised voles (*Myodes rutilus*). The algorithms utilize only DXA-derived measurements of fat mass (FM_{DXA}), lean mass (LM_{DXA}), bone mineral content (BMC_{DXA}), body mass ($Mass_{DXA}$ = FM_{DXA} + LM_{DXA} + BMC_{DXA}), and fat-free mass (FFM_{DXA} = LM_{DXA} + BMC_{DXA}). The FM equations have higher amounts of unexplained error and varying results for free-living vs. lab-raised voles, as opposed to the FFM equation which shows very little error and is consistent for all groups of animals (Fig. 2.4c). This is consistent with the result that the FM_{DXA} measurement is less precise than the LM_{DXA} and BMC_{DXA} measurements which together comprise the FFM_{DXA} measurement (Table 2.1). Percent fat is most accurately determined using the FFM parameter to determine percent fat by difference (Fat_{Diff} = 100% - %FFM).

Predictive Equation	n	R^2	SEE (g)
$Mass_{GRAV}$ = 0.94($Mass_{DXA}$) – 0.16	22	1.00	0.41
TM = 1.13(BMC_{DXA}) + 0.14	22	0.82	0.11
TBW = 0.76(LM_{DXA}) + 0.12	22	0.98	0.60
TP = 0.20(LM_{DXA}) - 0.17	22	0.88	0.38
LM = 0.97(LM_{DXA}) + 0.14	22	0.98	0.64
FM = 0.90(FM_{DXA}) - 0.42	20	0.84	0.88
$FM_{Free\text{-}living}$ = 0.34(FM_{DXA}) + 0.02	9	0.65	0.09
$FM_{Lab\text{-}Raised}$ = 0.63(FM_{DXA}) + 1.24	11	0.80	0.75
FFM = 0.97(FFM_{DXA}) + 0.13	22	0.98	0.65
Fat_{Diff} = $Mass_{GRAV}$ – FFM			

Table 2.3 Effect of PIT Tags on DXA Measurements. The effect of subcutaneous PIT tags on direct DXA measurements of fat mass (FM), lean mass (LM), bone mineral content (BMC), and bone mineral density (BMD) in free-living voles is shown. Means and standard errors of the mean are reported. A paired t-test was used to compare the DXA measurements of voles (n=38) that were scanned while tagged and then while untagged with slight repositioning between scans. PIT tags caused a significant ($p<0.05$) mass increase in all DXA parameters, but the corrective terms can be applied to obtain safe, reliable measurements in tagged, sedated voles. To acquire an accurate measurement for each parameter, DXA values obtained from tagged animals must first be corrected using these terms before using any of the predictive equations in Table 2.2.

DXA Measurement	$Mean_{Tagged}$	$Mean_{Untagged}$	Paired Mean Diff.	p-value	Corrective Term
FM (g)	2.5±0.15	2.4±0.15	0.1	0.038	-0.1
LM (g)	17.9±0.81	17.7±0.81	0.1	0.046	-0.1
BMC (g)	0.624±0.034	0.550±0.034	0.074	<0.001	-0.074
BMD (g/cm^2)	0.0699±0.0014	0.0609±0.0018	0.0090	<0.001	-0.0090

2.9 Figures

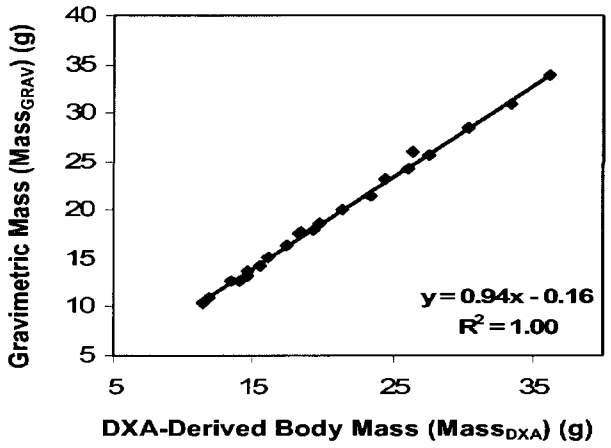

Figure 2.1 Gravimetric Mass Versus Dual-Energy X-ray Absorptiometry (DXA) - Derived Body Mass (Mass$_{DXA}$). The Mass$_{DXA}$ is the sum of the DXA-derived parameters of fat mass, lean mass, and bone mineral content. The DXA apparatus overestimates gravimetric body mass (massgrav) slightly but with virtually no unexplained error ($R^2 = 1.00$).

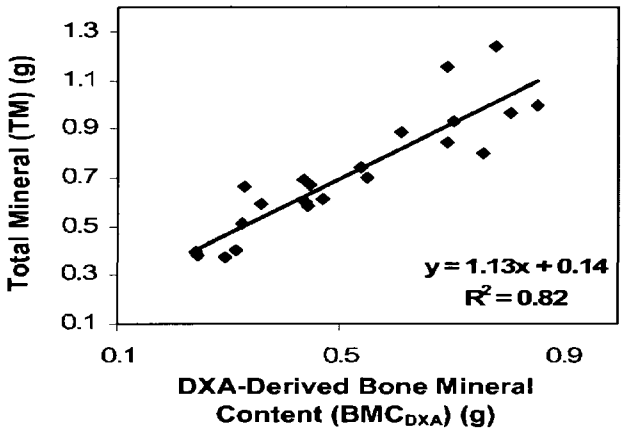

Figure 2.2 Total Mineral (TM) Versus Dual-Energy X-ray Absorptiometry (DXA) – Derived Bone Mineral Content (BMC_{DXA}). DXA predicts TM with a good amount of error explained by the resulting equation. The internal accuracy of the Lunar PIXImus BMCDXA measurement has already been affirmed (Nagy and Clair, 2000).

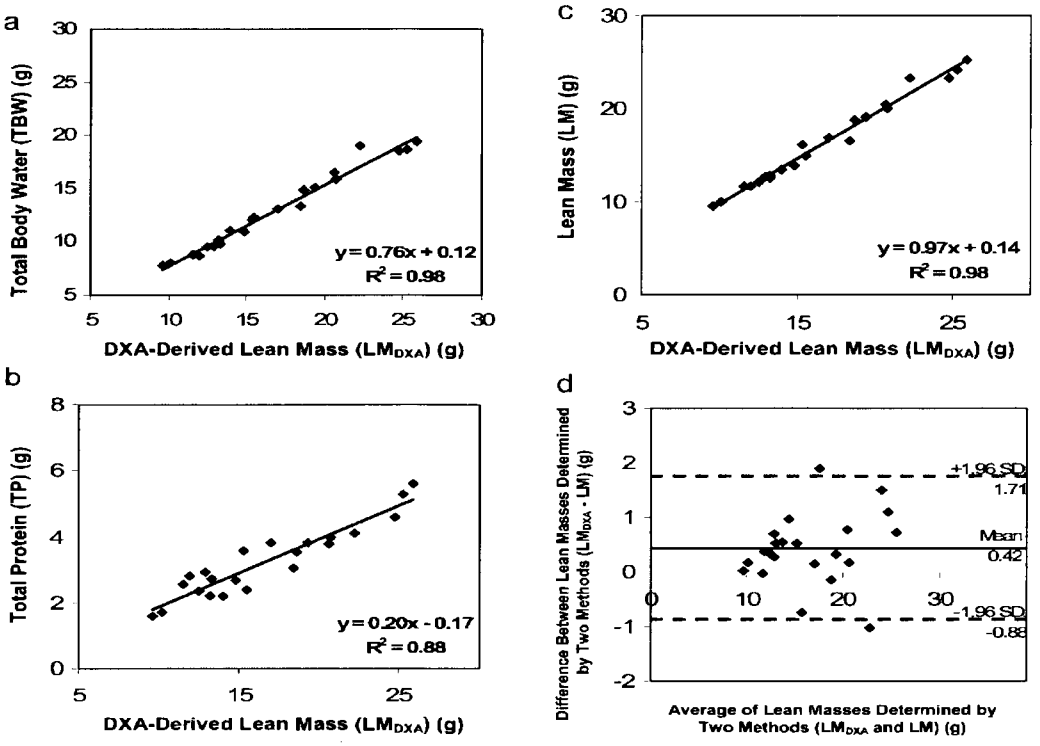

Figure 2.3 DXA-Derived Lean Mass Components. (*a*) Total body water (TBW) versus dual-energy X-ray absorptiometry (DXA) - derived lean mass (LM_{DXA}); (*b*), total protein (TP) versus LM_{DXA}; (*c*) lean mass (LM) versus LM_{DXA}; (*d*) Bland-Altman plot comparing two methods for determining LM. Water is the major component of both LM and fat-free mass (FFM), and the strong relationship between TBW and LM_{DXA} (R^2 = 0.98) (a) is tightly linked to the predictive equations for LM (c) and FFM (b). Fig. 2.3d compares the two methods used in determining lean mass, in which the difference is plotted against the average of the two methods to show sufficient agreement between them (Bland and Altman, 1986). The solid line is the mean difference, and the dashed lines represent 2 s.d. from the mean difference (95% limits of agreement).

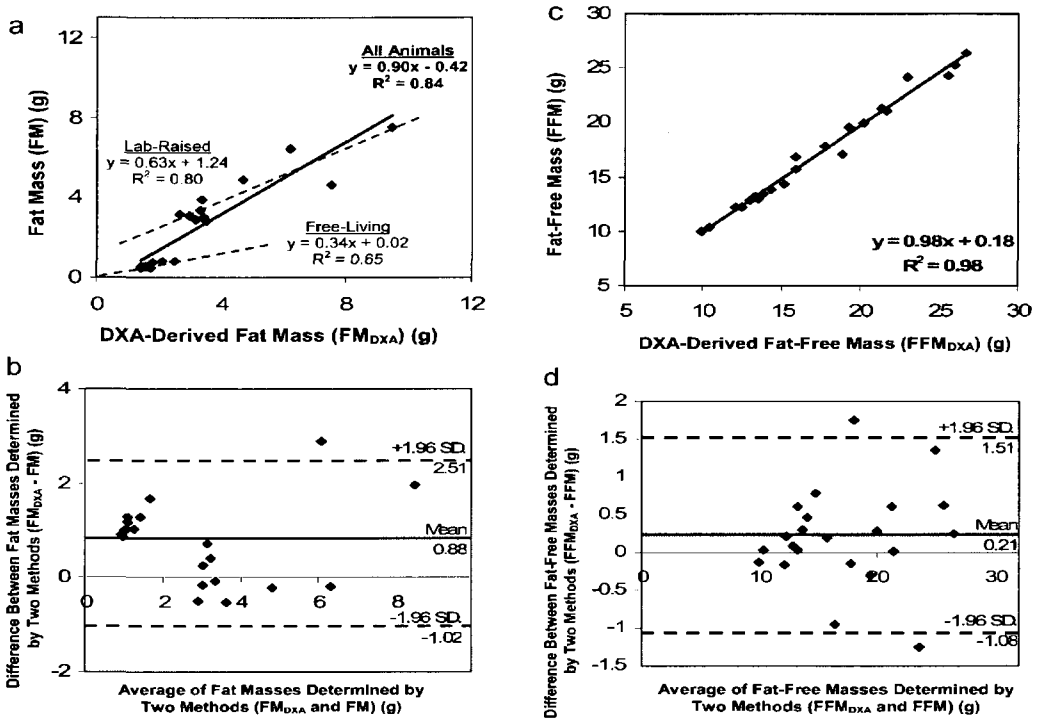

Figure 2.4 DXA-Derived Fat Mass and Fat-Free Mass Components. (*a*) Fat mass (FM) versus dual-energy X-ray absorptiometry (DXA) - derived fat mass (FM_{DXA}) in free-living voles, in lab-raised voles, and in all voles grouped collectively; (*b*) Bland-Altman plot comparing two methods for determining fat mass; (*c*) fat-free mass (FFM) versus DXA-derived FFM (FFM_{DXA}) in all voles; (*d*) Bland-Altman plot comparing two methods for determining FFM. Predictive equations for FM explained a fair amount of error in free-living voles (lower dashed line; $R^2 = 0.65$) and a good amount of error in both lab-raised voles (upper dashed line; $R^2 = 0.80$) and all voles grouped collectively (solid line; $R^2 = 0.84$). Fig. 2.2c describes the excellent relationship between FFM and FFM_{DXA} ($R^2 = 0.98$) that could be used to determine FM by difference. The two different methods used in determining FM and FFM (DXA analysis and chemical analysis) are compared in Figs. 2.2b and 2.2d, respectively. In each graph, the difference is plotted against the average of the two methods to show sufficient agreement between them (Bland and Altman, 1986). The solid line is the mean difference, and the dashed lines represent 2 s.d. from the mean difference (95% limits of agreement).

Chapter 3:

The Seasonality of Reproduction in Photoperiod Responsive and Nonresponsive Northern Red-backed Voles (*Myodes rutilus*) in Alaska[1]

3.1. Abstract

High-latitude arvicoline rodents usually reproduce in warmer months, but winter breeding has been documented in several species, including the northern red-backed vole (*Myodes rutilus*). I tested whether this species' reproductive condition is linked to changes in environmental parameters or its body condition, and I tested the frequencies at which different reproductive phenotypes are exhibited under field and laboratory conditions. Free-living voles in southcentral Alaska reached peak reproductive organ masses in spring (females) and early summer (males). Between-subject comparisons showed an effect of body mass, photoperiod, percent fat, temperature, and snow depth on reproductive organ masses, depending on the sex and breeding period ($p < 0.05$). One instance of late-summer photoperiod non-responsiveness was observed, but I detected no winter breeding. Captive male voles given food *ad libitum* and housed at room temperature exhibited strong phenotypic variation in testis mass in response to short photoperiods. The percentage of non-responders was 28.2% and within the known range of non-responsiveness for lower-latitude species (20-40%). Thus, photoperiod non-responsive morphs are conserved in at least one arctic/subarctic species at frequencies comparable to lower-latitude voles despite no observance of winter-breeding in the field. Voles exhibit reproductive plasticity and may breed in winter if environmental conditions enable them.

[1]Stevenson KT, van Tets IG, Nay LAI. 2009. The seasonality of reproduction in photoperiod responsive and nonresponsive northern redbacked voles (*Myodes rutilus*) in Alaska. *Can J of Zool* 87:152-164.

3.2 Introduction

Arvicoline rodents (voles and lemmings) are small mammals that strongly influence plant growth and distribution in Northern ecosystems (Schultz, 1964; Pitelka, 1964; Howe and Brown, 1999) and that form a major prey resource for predatory birds and mammals (Wilson and Bromley, 2001; Gilg *et al.*, 2003). In non-tropical species, reproduction is usually triggered by day length and is linked to other environmental variables. The high energy costs of winter thermoregulation and summer reproduction create an energetic bottleneck that shapes the seasonality of breeding (Levins, 1968; Nelson *et al.*, 1998). Reproduction usually occurs during warmer months and longer day lengths in most temperate-zoned and high-latitude arvicoline rodents (Sealander, 1967; Batzli and Pitelka, 1971; Nelson, 1985b; Hasbrouck *et al.*, 1986; Negus and Berger, 1998; Wallen and Schneider, 2000; McNabb, 2002). It ceases prior to winter and is usually characterized by regression of reproductive organs. Yet, some are able to overcome these reproductive challenges and constraints to successfully breed out of season. Amazingly, almost every species studied has been documented to have bred in winter (Nelson, 1987; Bronson, 1989; Bronson and Heideman, 1994; Nelson *et al.*, 1998; Gockel and Ruf, 2001) – even arctic/subarctic voles and lemmings (Mullen, 1968; Whitney, 1976; Eriksson, 1984; Hansson, 1984; Millar, 2001). Winter-breeding is very rare, however, and does not occur in all individuals or all years, suggesting an environmental or nutritional influence upon its frequency. Photoperiod non-responsive morphs are unique individuals within small mammal populations that possess the genetic capability to maintain the size and function of their reproductive structures under short day lengths (Nelson, 1987; Kriegsfeld *et al.*, 2000a). In lower latitude vole colonies, approximately 20-40% of individuals housed at room temperature and given food and water *ad libitum* exhibit the non-responsive phenotype when exposed to short days (Nelson, 1987; Kriegsfeld *et al.*, 2000a). Non-responsiveness has not been examined closely in arctic or subarctic species.

Frequencies of winter breeding have not been compared across a latitudinal gradient, but it seems plausible that there could be selection against the photoperiod non-responsive trait (*i.e.*, winter breeding ability) in high-latitude regions that might contribute to a decrease in its frequency. Winters are harsher, and individuals that attempt to breed in winter could be less likely to survive and/or have their offspring survive. This might cause a decrease in the frequency of non-responsive morphs in small mammal populations at high-latitudes.

Reproduction is an expensive process for arvicoline rodents, and the timing of breeding is critical for maximizing reproductive fitness. The costs of gestation and lactation can more than double total energy requirements in voles (Kacmarski, 1966; Migula, 1969; Millar, 1977; Innes and Millar, 1981), and they can triple or quadruple energetic costs in other mammalian taxa (Bronson, 1989; Clutton-Brock, 1991). Both male and female arvicoline rodents live a much faster pace of life in spring and summer through increases in mating interactions, activity, foraging, and growth rates (Stebbins, 1974; Batzli and Esseks, 1992; Zuercher *et al.*, 1999; Getz *et al.*, 2005). In fact, voles born in spring have been known to reach maturity and produce litters by the end of summer in the same year (Whitaker, 1996).

In winter, voles do not migrate or hibernate, but remain active under the snow (Sealander, 1967; Stebbins, 1974; West, 1977). They occupy the subnivean space and employ changes in their body mass, overall physiology, social structure, and activity to save energy (Sealander, 1967; Sealander, 1972; Stebbins, 1974; West, 1977; Wolff and Lidicker, 1981). Despite these seasonal changes in physiology and behavior, voles and lemmings must still use large amounts of energy to maintain a relatively constant body temperature via shivering and non-shivering thermogenesis. Voles that maintain reproductive structures to breed in winter are also likely to maintain body conditions conducive to fueling and/or maintaining excess reproductive tissues, mating interactions, and offspring.

Non-photoperiodic cues, such as temperature, precipitation, food availability, and the ingestion of plant secondary compounds in green vegetation have been proposed and/or affirmed as alternate signals cuing for reproduction in species inhabiting seasonal environments (Negus and Berger, 1977; Negus and Berger, 1998; McNabb, 2002), but photoperiod has been regarded as the dominant cue (Nelson, 1987; Kriegsfeld and Nelson, 1999; Wallen and Schneider, 2000; McNabb, 2002). Males respond to changes in photoperiod (Sealander, 1967; Whitney, 1976; Batzli and Pitelka, 1971; Nelson, 1985a), but the cause of female readiness in the wild is less well known and may be species specific. Females may respond to one or more environmental variables such as photoperiod (Meek and Lee, 1994), food availability, or temperature, but they are more commonly thought to respond to stimulation from a reproductive male (*e.g.*, copulation or some olfactory cue – Breed, 1972; Odberg, 1984; Clulow and Mallory, 1970).

The effect of body condition or nutritional state on the reproductive timing and condition of arvicoline rodents is also not entirely known. Previous studies have shown an influence of nutrition and food availability on reproductive state (Cengel *et al.*, 1978; Hasbrouck *et al.*, 1986), but body fat levels do not necessarily correlate with reproductive condition (Batzli and Esseks, 1992) and may or may not change seasonally (Cengel *et al.*, 1978; Batzli and Esseks, 1992; Zuercher *et al.*, 1999).

The primary aim of this study was to determine the timing of reproduction, the pattern of organ mass change, the factors affecting timing and seasonal change, and the frequency of photoperiod non-responsiveness in the northern red-backed vole, *Myodes rutilus* (Wilson and Reader, 2005; formerly *Clethrionomys rutilus*), an arvicoline rodent that inhabits both subarctic and arctic climates and is known to have bred in winter (Khlebnikov, 1970; Whitney, 1976; Whitney, 1977; Hansson, 1984). Maximum organ size is often correlated with peak reproductive activity in small mammals (Brown, 1997), while decreases in reproductive organ masses correspond to declines in breeding (Benton, 1955). Therefore, I trapped

voles at regular intervals over multiple seasons in southcentral Alaska and dissected them to test for correlations between reproductive organ masses and environmental and body condition parameters. In the aforementioned studies that report reproductive data for *M. rutilus*, occurrences of winter-breeding were moderate at a lower latitude site in the Western Sayan Mountains near the Russian-Mongolian border (Khlebnikov, 1970), but very rare at higher latitude sites in interior Alaska (Sealander, 1967; Whitney, 1976; Whitney, 1977; Hansson, 1984). I predicted that in coastal southcentral Alaska (61°N), out-of-season breeding would be more frequent than in interior Alaska due to longer winter day lengths, relatively milder temperatures, more abundant ground-level vegetation, and generally higher levels of snowfall. Finally, I used a controlled, captive experiment to test whether the frequency of non-responsive morphs in this high-latitude species is conserved relative to temperate-zone species, and I tested my hypothesis that a relationship exists between reproductive phenotype and body mass.

3.3 Methods

3.3.1 Field Study

Northern red-backed voles were collected from trapping grids around Chugach State Park, Alaska (61°N, 150°W) between November 2004 and August 2006. Animals were captured and treated following procedures approved by the University of Alaska Anchorage Institutional Animal Care and Use Committee (IACUC) committee (protocol #2005vanTe1). The voles' breeding condition was first assessed externally by the presence of descended testes in males and by palpation, occurrence of lactation, or presence of open pubic sympheses in females (Batzli and Pitelka, 1971). We dissected and weighed the testes, epididymides, and seminal vesicles of males and the ovaries and uterus of non-pregnant females. Seven of the females trapped during the study were in varying stages of pregnancy. Their paired ovary masses were included in the analyses, but

their uterine weights were excluded. Males' testes were examined histologically to confirm the reproductive and non-reproductive state of these animals.

I used mass thresholds to distinguish age classes in *M. rutilus* in early and late summer seasons (Sealander, 1967; Whitney, 1976; Zuercher *et al.*, 1999). In summer (June through September), voles weighing <13g were considered immature juveniles, those ranging from 13-17.5g were classified as adolescents, and those >17.5g were determined to be of adult age (Zuercher *et al.*, 1999). In the remaining seasons, all animals were classified as adults.

The compiled reproductive organ mass data set was bimodal due to the short transition period between non-reproductive and reproductive periods. Therefore, I divided the compiled reproductive organ mass data into two subsets corresponding to a general breeding period (April 1st to August 25th) and a general non-breeding period (August 26th to March 31st). The reproductive data in each of the resulting subsets were log transformed to achieve normality. Fat mass was determined by scanning each animal with a dual-energy x-ray absorptiometry (DXA) apparatus (Lunar/GE PIXI Mus2) that was previously validated for use in *M. rutilus* to assess body composition (Stevenson and van Tets, 2008). The equation for fat mass in free-living voles from this validation was used to obtain actual fat mass values.

Photoperiod (hours of day light) was recorded on each capture date. Average ambient temperature (T_a) was recorded every hour by data loggers (Hobo, Inc.) that were placed at a height of 2m near my trapping sites. We used data from National Resources Conservation Services (NRCS) snowpack telemetry (SNOTEL) sites in Chugach State Park, Alaska (Anchorage Hillside) and Moraine, Alaska to confirm ambient temperature measurements and obtain levels of snow depth and precipitation. SPSS (v14.0) statistical software was used for all statistical analyses. We used analysis of covariance (ANCOVA) to determine between-subjects effects of body condition and environmental variables on reproductive organ masses, and I used simple regressions to characterize the

relationship between photoperiod and body condition.

3.3.2 Lab Study

From 2006 to 2007 I bred and raised captive *M. rutilus* from a pre-existing captive northern red-backed vole colony in animal quarters at the University of Alaska Fairbanks campus (IACUC protocol #06-53). The colony was composed of animals trapped in different locations around interior Alaska in 1999 and 2004, and was diverse in age and activity pattern (Tavernier *et al.*, 2004). I selected a subset of captive male voles that had been raised and maintained on long photoperiods (16L:8D). These voles were then age-matched and categorized into three groups: Pre-study control ('PRE', 16L:8D, $n = 20$), continued long day treatment ('LD', 16L:8D, $n = 18$), and short day treatment ('SD', 8L:16D, $n = 78$). Based on earlier work, I elected to place substantially more animals in the SD group to increase the chances of detecting non-responsive morphs in the event that the percentage of this morph was low and to assess the frequency of all three morphs with strong confidence (Kriegsfeld *et al.*, 2000a; Kriegsfeld *et al.*, 2000b). Approximately 30 days prior to the study, male subjects were separated from the colony and placed in individual cages. LD and PRE cages were kept on racks in a photoperiod-controlled room, while SD cages were kept in photoperiod-controlled chambers (up to 12 animals per chamber). All subjects remained on long photoperiods (25°C) at room temperature during this time and were given food and water *ad lib*.

Prior to the start of the study while all animals were on long days, I measured the external testis volume and body mass of each animal. We anesthetized voles using an isoflurane vaporizer interfaced with a plastic holding container and a re-breathe bag. We weighed each animal, shaved the left side of the scrotum, and obtained a left testis length and width (to the nearest 0.01 mm) using digital calipers. Testis volume was measured every 2 weeks, at which time a retro-orbital blood sample was collected from a subset of only 10 animals for use in a different

study. The technique is a standard laboratory procedure (Halpern and Pacaud, 1951; Hoff, 2000) and caused no harm, as voles were already anesthetized to obtain measurements of testis size. Blood samples were taken within 2 minutes of initial handling and did not add additional stress to the animals. Because of the large sample size, not every animal was bled during the 12- week study, and no animal was bled more than once.

At the start of the experiment, all PRE animals were euthanized for comparisons with the other groups at the end of the experiment, and served as a control group with respect to LD animals. An overdose of sodium pentobarbital (50 to 75mg/kg) was injected interperitoneally, and a toe-pinch reflex test was used to ensure that animals were unconscious. This allowed the heart to continue beating for several minutes while blood was drawn by cardiac puncture. Voles were then perfused with a phosphate-buffered wash solution and a 4% paraformaldehyde solution. Testes were removed, along with blood and other tissues for use in a separate study. We determined paired testis mass and actual testis volume of each subject. A strong correlation existed between perfused testis mass and both perfused testis volume and the estimated external testis volumes at week 10, ensuring that perfusion did not affect the integrity of the testis mass data.

The 12-week photoperiod treatment then commenced for the remaining two groups. LD animals remained on long photoperiods, while SD animals were switched to short photoperiods. All animals remained on *ad lib.* food and water at room temperature. After 12 weeks, LD and SD animals were euthanized in the same manner as PRE animals.

Based on final testes mass of PRE and LD animals, the twelve additional weeks of long day photoperiod treatment did not cause LD animals to become photorefractory (a regression of the testes due to overexposure of long days) relative to PRE animals that were euthanized twelve weeks earlier (t-test $t_{36} = 0.719$, $p = 0.477$). Only one LD animal had regressed testes, but this did not cause

any significant difference between the groups. Therefore, these two groups were combined into one large group ('LD*', $n = 37$) for testis mass comparisons with SD animals.

Means and standard deviations of LD* paired testis masses were calculated to determine the different photoperiodic morphs within SD animals (Nelson *et al.*, 1989; Kriegsfeld and Nelson, 1999). Any SD animal having a perfused paired testis mass within 1 standard deviation of the LD* mean was categorized as a photoperiod non-responsive morph (NR). Those falling between 1 and 2 standard deviations below the LD* mean were classified as intermediately responsive morphs (IR), and those having a testis mass below 2 standard deviations of the LD* mean were photoperiod responsive morphs (R). We used a t-test to assess differences between LD and SD animals, and I used analysis of variance (ANOVA) with Tukey's HSD tests to determine body mass differences between LD, NR, IR and R groups.

3.4 Results

3.4.1 Field Study

Although trapping was conducted on a consistent basis at different locations throughout Chugach State Park, animal capture was not always consistent. Summer trapping success typically ranged from 10-40%, while winter trapping success was usually less than 5%. Our most complete and consistent data set was from May 2005 to September 2006. Locating voles in winter was challenging, even when subnivean nest boxes were used. Animals trapped in fall and winter were often found in pairs or groups (West, 1977; Stevenson *et al.*, 2009), suggesting communal nesting.

Males in this study typically became reproductive and had descended testes in early spring (April). In spring of both 2005 and 2006, male voles showed increases in testis mass while snow still covered the ground and no grasses had emerged. In April 2006, I observed males becoming active through a steady

increase in reproductive organ mass from mid- to late-April. Voles were inactive through the end of March, but began to transition within the first 10 days of April. At this time, I also observed an external anatomical change in the female vole reproductive anatomy prior to any copulation with males. Females trapped during the same time as these males with increasing testis masses did not yet have open pubic sympheses. However, the presence of a small, thin line running laterally across the closed (unpenetrated) pubic symphesis of females was observed in mid-April. We did not detect this in voles trapped during the preceding winter, and suspect that it is an external indicator of an internal physiological change towards reproductive readiness. We detected evidence of copulation in females approximately 10 days after discovering this feature.

In spring and summer seasons, reproductively active males had large testes with expanded seminiferous tubules that contained a well-defined lumen and maturing (pinched) spermatids and spermatozoa. Inactive males had small testes with seminiferous tubules that lacked lumena, with no evidence of maturing spermatids or spermatozoa. In general, adult reproductive structures of males and females were largest in spring and early summer months. Organ masses declined at the end of summer and stayed low until the end of winter (Fig. 3.1). Litter size ranged from 4 to 7 pups and averaged 5.7 ± 0.4 pups per litter. No winter breeding was detected in the free-living populations studied.

Of the 13 adult male voles collected between August 22nd and September 14th, 2005, nine were inactive and had regressed testes (69.2%), three appeared to be regressing towards inactivity (23.1%), and one remained active (7.7%). Thus, twelve were responsive or intermediately responsive to photoperiod, while only one was found to be non-responsive and fully reproductive during this time (Table 3.1). The non-responsive animal had the largest body mass, the second largest amount of total body fat, and the largest value for testis: body mass index (Table 3.1). No reproductive males were observed later than this, and lactating females were not observed past October.

The ANCOVAs detected a significant effect of body mass, photoperiod, and temperature upon male reproductive organ masses during both the breeding and non-breeding periods ($p < 0.05$, Table 3.2a). In females, body mass, percent fat, photoperiod, and snow depth all had a significant effect upon female reproductive organ masses during the non-breeding season only (Table 3.2b). Both body mass and total fat mass were positively correlated with photoperiod, but percent fat remained low year-round and did not change seasonally (Fig. 3.2).

3.4.2 Lab Study

We observed a high degree of intraspecific variability in testis mass within the SD treatment group after the 12 week photoperiod treatment (Fig. 3.3). All three reproductive phenotypes were exhibited, and in the following proportions (mean paired perfused testis masses ± SEs are in parentheses): 28.2% NR (0.5457 ± 0.0137, $n = 22$), 20.5% IR (0.4135 ± 0.0095, $n = 16$), and 51.3% R (0.1397 ± 0.0145, $n = 40$). Older and younger animals were well-represented in each reproductive grouping. The overall change in body mass was significantly higher in the LD group than both the IR group (-0.631 ± 0.672, $p < 0.001$) and the R group (-4.688 ± 0.812, $p < 0.001$), a trend that mirrored changes in testis mass (Fig. 3.3). Overall, SD animals exhibited a negative change in body mass over twelve weeks, while LD animals exhibited a slight mass increase (Fig. 3.4), and the difference between the two groups was significantly different (t-test $t_{94} = 4.459$, $p < 0.001$). The mean change in body mass over the course of the study was highest in the LD group (2.917 ± 0.675), but this was not significantly different from the mass change in the NR group (0.650 ± 0.612, ANOVA multiple comparisons, $p = 0.283$, Fig. 3.4).

3.5 Discussion

Arctic and subarctic voles are not frequent winter breeders in the wild but do maintain the ability to breed out-of-season. This is achieved through the presence

of non-responsive morphs within their populations. The frequency of non-responsive morphs in captive *M. rutilis* fell within the known range for lower latitude voles, suggesting no selection against the morph at high latitudes. Non-responsive animals were typically in better condition (larger body mass, more total body fat, larger testis: body mass index) than responders, as observed in my field and laboratory studies. Reproductive organ masses of males and females were linked to environmental variables and body mass, but not body fat percentage.

The reproductive organ masses of *M. rutilus* in southcentral Alaska showed dramatic seasonal changes similar to those recorded for voles in interior Alaska (Sealander, 1967; Whitney, 1976; Fig. 3.1). The longer winter photoperiod, milder coastal climate, abundance of low-level vegetation, and heavier snowfall characteristic of southcentral Alaska did not contribute to higher levels of photoperiod non-responsiveness or winter-breeding relative to these earlier studies in the Interior. Although southcentral Alaska experiences higher levels of snowfall, it can be susceptible to unpredictable freeze-thaw cycles in fall, winter, and spring. We observed such events in each non-reproductive season in every year of the study. These freeze-thaw cycles are likely to negatively affect vole survival and inhibit winter breeding. In higher-latitude and non-coastal regions where freezing and thawing are usually predictable and relatively isolated seasonal events, survival and reproduction of arvicoline rodents appear to be less affected by extremely low winter temperatures and low snowfall, but more negatively affected by first snowfall and spring melt (Whitney, 1976; Millar, 2001). This is probably due to the presence of low temperature water (freezing rain, sleet, melted snow) that accumulates and can increase the rate of heat loss from voles through conduction. Water that re-freezes into sheets of ice may also inhibit foraging and energy intake at this time, further reducing chances for survival and reproduction . However, Holleman *et al.* (1982) found that field metabolic rates of free-living *M. rutilus* in interior Alaska were higher in winter

and summer than in spring, suggesting that extremely low temperatures and extremely high temperatures might adversely affect vole survival more than spring melt in that region.

Both male and female voles became reproductive at the beginning of April under an increasing photoperiod of ~13h of daylight and began to regress at the end of August under a decreasing photoperiod of ~ 14.5h of daylight (Fig. 3.1). In consecutive years, voles became reproductive in April while snow still covered the ground and grasses had not yet emerged. This indicates that voles in this region do not typically time reproduction through plant secondary compounds (see Negus and Berger, 1977; Berger *et al.*, 1981; Negus and Berger, 1998) because they begin their reproductive change well before any freshly sprouted monocots are available for them to ingest. Body mass and bone mineral density in this species begin to increase at the end of late winter and rapidly increased in early spring (Stevenson *et al.*, 2009; Fig. 3.2). The presence of hypogeous fungi in the stomachs of voles and on the roots of birch and other trees in different seasons suggest that this could be a primary food source that helps to fuel the increases in body mass and reproductive organ masses in spring. Thus, a readily available food source may be present to fuel winter-breeding, but access to it may be limited to the extent that any autumnal or late winter freeze-thaw cycles can generate ice sheets.

The voles that I studied did not show seasonal changes in body fat percentage (Fig. 3.2c), but instead underwent increases in total body fat (Fig. 3.2b) and total body mass (Fig. 3.2a) during the reproductive season (similar to Zuercher *et al.*, 1999). Although fat percentage remained low year-round, more total fat was available in spring and summer for breeding activity, territory guarding, and/or gestation/lactation (Fig. 3.2b). It appears to be immediately replenishable by the high availability of preferred foods in the surrounding environment during the breeding season. The ability of non-responsive morphs in northern red-backed vole and other arvicoline rodent populations to maintain, or even increase body

size or body fat in a given winter (Anderson and Rauch, 1984; Batzli and Esseks, 1992; Zuercher *et al.*, 1999) could determine their ability to breed during that season.

The level of diversity in this high-latitude species' reproductive response to photoperiod is profound in that it is likely to allow for continued breeding during reduced photoperiods when environmental or nutritional conditions are favorable. Whether non-responsive morphs exhibit the non-responsive phenotype and attempt to breed in winter during a given year may ultimately be related to energy availability and expenditure. Specifically, there is likely to be an effect of food availability, food quality, or temperature on photoperiod non-responsiveness. For instance, almost all male prairie voles (*Microtus ochrogaster*) housed at 8°C undergo testicular regression in response to short photoperiods, while those housed at 20°C on identical day lengths exhibit the normal 20-40% frequency of non-responsiveness (Kriegsfeld *et al.*, 2000a). Food supplementation and photoperiod have been shown to interact to influence the reproductive condition of pine voles (*Microtus Pitymys pinetorum*), suggesting a strong nutritional effect (Cengel *et al.*, 1978; Hasbrouck *et al.*, 1986).

It seems logical that winter breeding would usually occur by an extension of the late-summer breeding season (as shown by the one non-responsive adult male in my study that showed no regression of testes during late summer). However, another factor (*e.g.*, warm temperatures or acute food availability) could cause the spontaneous growth of reproductive structures in fall or mid-winter, and subsequent out-of-season breeding would commence. Two studies on record support the idea that spontaneous mid-winter recrudescence of reproductive structures is possible. Kaikusalo and Tast (1984) observed a population of root voles in Kilpisjarvi, Finnish Lapland (69°N, 21°E) in which none of the trapped subjects were reproductively active in October. When the same population was re-trapped in February, however, 80% were found to be breeding. Similarly, Whitney (1976) found no early-winter breeding in *M rutilus*, yet he found one

individual in late winter (March) that was in reproductive condition. An alternate explanation for these occurrences is that there may be some natural selection occurring for an early entry into reproductive condition based on a day length cue that is shorter than the normal critical day length that typically triggers reproductive activity for each species (both occurrences were between the winter solstice and spring equinox when days were getting progressively longer). Each of these studies occurred at latitudes higher than 64° N where February and March temperatures often reach below -40°C. However, unseasonably high temperatures or a particularly snowy winter generating a thick insulative snowpack could lower overall costs of thermoregulation.

Whether the rare occurrences of winter-breeding in small mammals are attributed to an extension of the normal breeding season or to spontaneous mid-winter recrudescence of reproductive structures, an acute energy source is likely to be beneficial for males, females, and offspring. Moose and other large animals often starve, fall ill, or are killed by other animals or humans prior to or during winter. If the whole or partial carcass of such an animal became buried by snowfall, it could provide a protected, long-term, nutritious, subnivean food source for a local population of voles or lemmings that might, thereby, enable non-responsive morphs to maintain or re-enter a reproductively active condition and breed successfully. *M rutilus* may be primarily herbivorous, but it will feed on animal remains in winter. Snap-trapped voles at my study site sometimes were cannibalized by other voles, and hair from various mammalian species was found in the feces and stomach contents of winter-trapped *M. rutilus* (Stevenson and van Tets, pers. observ.) We found anecdotal support of this 'fallen moose hypothesis' in the form of a communal vole nesting area detected near a moose carcass I discovered northeast of Fairbanks, Alaska along the Chatanika River during the spring melt of 2007. The carcass was surrounded and partially covered by tens of thousands of fecal pellets of a size and shape characteristic of *M. rutilus* (Stevenson pers. observ.). A moose or caribou that starves in winter and is

covered by snow will not begin to decompose until spring. If 20-40% of a nearby vole population is genotypically non-responsive, a high rate of local winter breeding could result.

Captive *M. rutilus* showed a high degree of intraspecific variation in their response to short photoperiod (Fig. 3.3). Previous studies on lower latitude small mammal species have listed the percentage of non-responsive morphs in a population as either 20-30% (Eskes and Zucker, 1978; Zucker, *et al.* 1980; Beasley *et al.*, 1981; Nelson and Zucker, 1981; Desjardins and Lopez, 1983) or as 20-40% (Nelson, 1987; Kriegsfeld *et al.*, 2000a). The percentage of non-responsive individuals in the captive population of a high-latitude vole was 28.2%, within the expected range of frequency for captive lower latitude small mammal populations. This validates the sporadic observance of winter-breeding that has been detected in many high-latitude species. Despite the fact that winter breeding is rare in the arctic and does not occur in all individuals or all years, the frequency of non-responsive morphs in the colony appeared undiminished.

Long-term, established captive rodent colonies have sometimes selected for NR individuals, increasing the percentages of non-responders relative to those present in the wild (Gorman and Zucker, 1997; Nelson, 1985b). Such confusion is unlikely in this case, as I used only reproductively mature animals from a relatively young colony (started nine years ago, with free living additions as recently as five years ago) that has only ever been bred, weaned, and raised to age of reproduction on long photoperiods. Selection in at least one documented case was the result of breeding, weaning and juvenile development under short photoperiods (Nelson, 1985b). The frequency of non-responsiveness I observed (28.3%) was also within the typical range for lower latitude rodents (Nelson, 1987; Kriegsfeld *et al.*, 2000a).

The mechanisms underpinning winter-breeding have still not been entirely identified. However, body mass (but not necessarily percentage body fat) was correlated with reproductive state in both my field (Fig. 3.2) and captive studies

(Fig. 3.4), regardless of photoperiod. Gonadectomy is known to enhance body mass changes in other vole species (Kriegsfeld and Nelson, 1996), and gonadal mass and body mass appear to be linked in *M. rutilus* (Figs. 3.3 and 3.4). In my captive study, the direction and degree of change in gonadal mass and body mass were similar within each photoperiodic group. However, decreasing day lengths can arrest both somatic and reproductive development, and it is not impossible that photoperiod variably altered body mass, resulting in some effect upon reproductive condition. The uncoupling of photoperiodic processing and reproductive function has been studied in other small mammals. Mice (*Mus musculus*) process photoperiodic information, discriminating short from long days. While this is normally uncoupled from reproduction, the interaction of bulbectomy or testosterone injections with short days causes testicular regression (Nelson, 1990). Interestingly, body mass is not affected by day length in such cases, as it was for *M. rutilus* in my study. In *Peromyscus*, the daily rhythm of melatonin antigonadal action appears to be linked to changes in responsiveness of melatonin target neurons in the suprachiasmatic nucleus (SCN). As in many rodent species, an endogenous timing mechanism sustains reproductive inhibition in *Peromyscus* under short day lengths until photorefractoriness occurs, resulting in spontaneous testicular recrudescence (Glass and Lynch, 1982).

Photoperiodic sensitivity and insensitivity in Syrian hamsters, *Mesocricetus auratus,* has also been attributed to effects of melatonin produced in the pineal gland. Syrian hamsters measure day length by determining whether light impinges on an endogenous circadian rhythm of sensitivity to light. Reproductive stimulation occurs when day length is long enough to overlap with the sensitive phase of this endogenous rhythm (Elliot, 1981; Elliot and Goldman, 1981; Bronson, 1989). Conversely, reproductive inhibition occurs when day lengths are not long enough to achieve this. Strains of this species have been shown to vary drastically in their reproductive response to shortened photoperiods, and the length of short photoperiod exposure is known to have an effect on the degree of

testicular regression (Vitaterna and Turek, 1993). Yet, genetic differences in reproductive response to photoperiod do not appear to be linked to circadian rhythmicity for this species (Vitaterna and Turek, 1993).

The extent to which circadian rhythm and reproductive state are linked in voles is unknown. Differences may exist in melatonin production in the pineal gland or in the SCN, but it is quite possible that individuals first begin displaying differences further downstream at some point along the hypothalamic-pituitary-gonad axis, *e.g.*, expression of GnRH or GnIH neuropeptides in the hypothalamus, LH or FSH in the pituitary, or gonadal testosterone production.

The link between reproductive organ mass and body mass observed in my captive study (Fig. 3.4) sheds new light upon my field data (Table 3.2, Fig. 3.2a). It is likely that in free-living populations, many older animals die-off prior to or during winter because a larger body size is not typically conducive to survival in these seasons. The energetic costs of maintaining reproductive organ masses and continued breeding into the fall and winter seasons could negatively affect immune function and overall survival (Nelson and Demas, 1996), and a large body size could intensify or accelerate these negative effects in certain seasons. Perhaps this is why most overwintering animals are found with smaller body sizes and most larger animals die-off. Although a larger body size may, by virtue of its lower surface area: volume ratio, resist heat loss more effectively than a smaller one, the benefit of lower total tissue maintenance costs appears more important to winter survival in some arvicoline rodents. Non-responsive voles may, however, be able to take advantage of both a large body size and lower individual maintenance costs in winter through communal nesting (West, 1977; Wolff and Lidicker, 1981; West and Dublin, 1984, Stevenson *et al.*, 2009). Voles huddle together and become one larger 'vole unit' to resist heat loss, separating only to forage. Such communal nesting should improve the success of winter breeding through the close proximity of males and females. Since most small rodents have life spans of less than one year in the wild, the process of winter breeding could

significantly increase the fitness of non-responsive morphs. More data are needed to investigate cues and responses of female voles. Our field results suggest an effect of photoperiod on reproductive structures, but ovulation may ultimately be induced by males. It is possible that females also exhibit an intraspecies variation in their reproductive response to short photoperiods, and this should be tested further in more species (especially high-latitude ones).

We conclude that the combined data on reproductive timing and intraspecific variation in response to photoperiod portray this species as reproductively flexible. Therefore, if climate changes in this region in ways that reduce the energetic demands on voles during fall and winter, they are likely to be able to breed for longer lengths of time, regardless of shortened photoperiod. Conversely, if climate changes in ways that are more conducive to unpredictable freeze-thaw events and a reduced insulative snow pack, breeding is likely to be restricted to the summer period. Change in either direction in a vole or lemming population is likely to affect soil nutrient loads and vegetative maintenance (Schultz, 1964; Howe and Brown, 1999), the abundance of predators that rely heavily on rodents (Wilson and Bromley, 2001; Gilg *et al.*, 2003) and the secondary prey species subject to increased predation when voles are scarce (Sittler *et al.*, 2000; Wilson and Bromely, 2001).

3.6 Conclusion

Free-living male and female northern red-backed voles exhibit dramatic seasonal changes in their reproductive organ masses as a result of a photoperiodic threshold. Body mass changes proportionally to testis mass in male voles, and testis mass exhibits considerable variation in its reproductive response to short photoperiods in the laboratory. Highly seasonal environments inhibit the ability of most arvicoline rodents to breed in winter, but the apparently rare occurrence of winter reproduction has not resulted in a lower proportion of photoperiod non-responsive morphs relative to lower-latitude vole species. The diverse phenotypic

reproductive response of *M. rutilus* to short day lengths under laboratory conditions confirms that reproductive timing is fairly flexible in voles. Northern red-backed voles are able to breed successfully when good summer conditions are prolonged or when winter conditions and/or food availability become more favorable. A long term warming trend in northern latitudes could affect the frequency and timing of breeding of this and related species, and thus also vegetation, soil nutrient loads, and the success of predator and prey species that are influenced by them. Whether the effect of such a potential trend is positive or negative is likely to depend on its effect on the thickness of the insulatory winter snow pack and on climactic conditions in spring and fall.

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3.8. References

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3.9 Tables

Table 3.1 Reproductive Status of Adult (>17.5g) Male *Myodes rutilus* Trapped From 8/29 to 9/14 in 2005. Of the thirteen animals collected, nine were reproductively inactive, three appeared to be transitioning, and one was reproductively active. This single active animal had a larger body size and a larger testes-mass index.

Trap date	Body mass (g)	Fat mass (g)	Percent fat (%)	Paired testis mass (g)	Testes : body mass index	Reproductive state
29 August 2005	18.8	0.88	4.7	67	4	Inactive
	18.7	0.76	4.1	64	3	Inactive
30 August 2005	19.9	1.50	7.5	70	4	Inactive
	20.4	0.80	3.9	51	3	Inactive
	18.4	0.94	5.1	70	4	Inactive
	18.6	0.77	4.1	165	9	Regressing
	26.6	1.08	4.1	4563	172	Active
5 September 2005	19.3	0.93	4.9	108	6	Regressing
7 September 2005	17.6	0.61	3.6	51	3	Inactive
14 September 2005	18.4	0.82	4.5	60	3	Inactive
	19.1	0.99	5.2	83	4	Inactive
	19.4	0.70	3.6	284	15	Regressing
	19.4	0.81	4.2	69	4	Inactive

Table 3.2 Analysis of covariance (ANCOVA) of adult *Myodes rutilus* reproductive organ masses during breeding and non-breeding periods. *P*-values less than 0.05 are in bold. Epid. = Epididymides, S.V. = Seminal Vesicles, Ovar. = Ovaries, Uter. = Uterus. Uteri containing pups were excluded from analyses. Sample size (*n*) for breeding period: Males = 30, Females = 11 and 18 for ovarian and uterine masses, respectively. Sample size for non-breeding period: Males = 35, Females = 26.

	Male						Female			
	Log(testes mass)		Log(epididymides mass)		Log(seminal vesicles mass)		Log(ovaries mass)		Log(uterus mass)	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
(A) Breeding period										
Corrected model	**5.656**	**<0.001**	**16.764**	**<0.001**	**7.540**	**<0.001**	0.828	0.587	0.260	0.935
Intercept	0.376	0.546	3.404	0.079	1.973	0.174	1.173	0.304	0.958	0.400
Fixed factors										
Year	2.588	0.122	0.205	0.655	0.042	0.840	0.121	0.735	0.653	0.478
Covariates										
Log(mass)	**8.465**	**0.008**	**59.916**	**<0.001**	**13.281**	**0.001**	1.447	0.257	0.006	0.944
Percent fat	2.310	0.143	0.002	0.963	0.064	0.802	2.097	0.178	0.754	0.449
Photoperiod	0.851	0.366	**5.534**	**0.028**	0.028	0.869	0.006	0.938	0.988	0.394
Snow depth	0.635	0.434	1.118	0.302	0.648	0.429	0.364	0.560	0.472	0.541
Precipitation	0.374	0.547	2.914	0.102	0.220	0.643	0.066	0.803	0.843	0.426
Temperature	**5.555**	**0.028**	**14.408**	**<0.001**	0.715	0.407	0.570	0.468	0.813	0.434
(B) Nonbreeding period										
Corrected model	**2.579**	**0.036**	**3.420**	**0.010**	**2.584**	**0.035**	1.262	0.322	**5.003**	**0.003**
Intercept	**12.026**	**0.002**	**13.851**	**0.001**	**12.148**	**0.002**	2.468	0.134	**7.833**	**0.012**
Fixed factors										
Year	0.203	0.140	0.280	0.079	0.158	0.147	0.350	0.562	3.914	0.063
Covariates										
Log(mass)	**14.842**	**0.001**	**18.306**	**<0.001**	**14.937**	**0.001**	**5.530**	**0.030**	**21.718**	**<0.001**
Percent fat	1.603	0.216	2.734	0.110	1.614	0.215	0.748	0.399	**6.279**	**0.022**
Photoperiod	**7.096**	**0.013**	**8.720**	**0.006**	**7.451**	**0.011**	0.202	0.658	**5.082**	**0.037**
Snow depth	0.401	0.532	0.166	0.687	0.438	0.514	1.404	0.252	**12.489**	**0.002**
Precipitation	1.519	0.228	1.839	0.186	1.634	0.212	0.195	0.664	0.032	0.860
Temperature	**4.401**	**0.045**	**5.673**	**0.025**	**4.603**	**0.041**	0.120	0.733	1.544	0.230

Note: $P < 0.05$ are in boldface type. Uteri containing pups were excluded from the analyses. Sample size (*n*) for breeding period: males = 30; females = 11 and 18 for ovarian and uterine masses, respectively. Sample size for nonbreeding period: males = 35; females = 26.

3.10 Figures

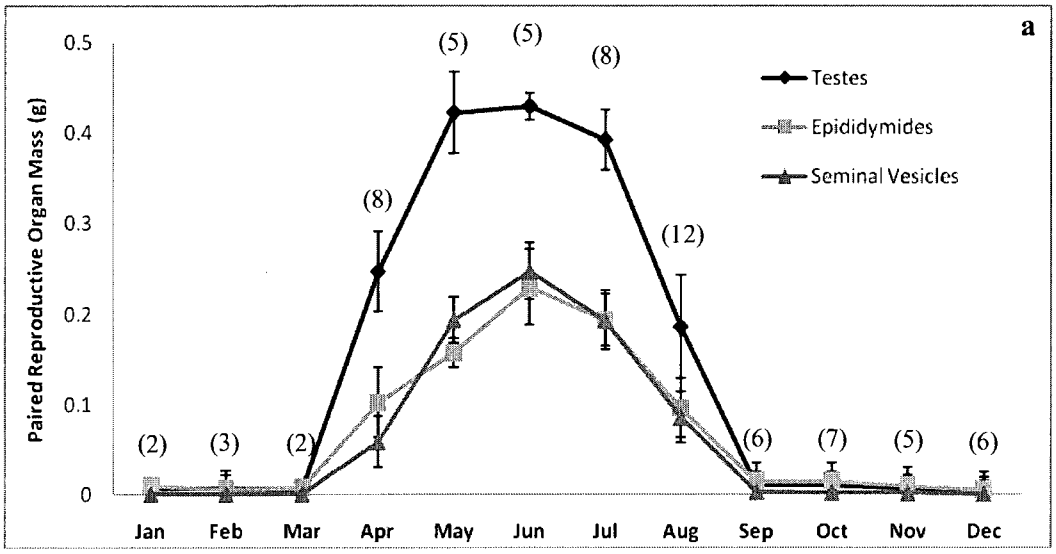
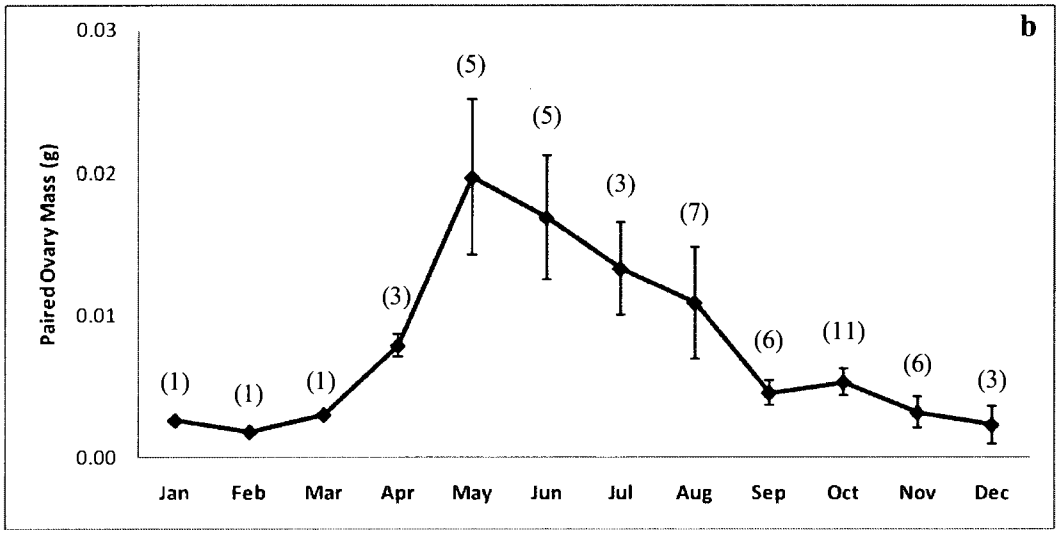


Figure 3.1 Monthly Variation in Reproductive Organ Masses of Male and Female *Myodes rutilus* in Chugach State Park, Alaska. Means and SEs are shown with sample sizes in parentheses. (*a*) Male testes, epididymides, and seminal vesicles of males increased early in spring, peaked in early summer, and declined in late summer. (*b*) Ovarian mass also reached peak values in spring and decreased gradually to their lowest levels in winter (*c*) Non-pregnant female uterine masses reached peak values in spring and decreased during late summer. Only non-pregnant uterine masses were used in summer months, and this is reflected in the lower sample sizes for these months relative to Fig. 3.1b.



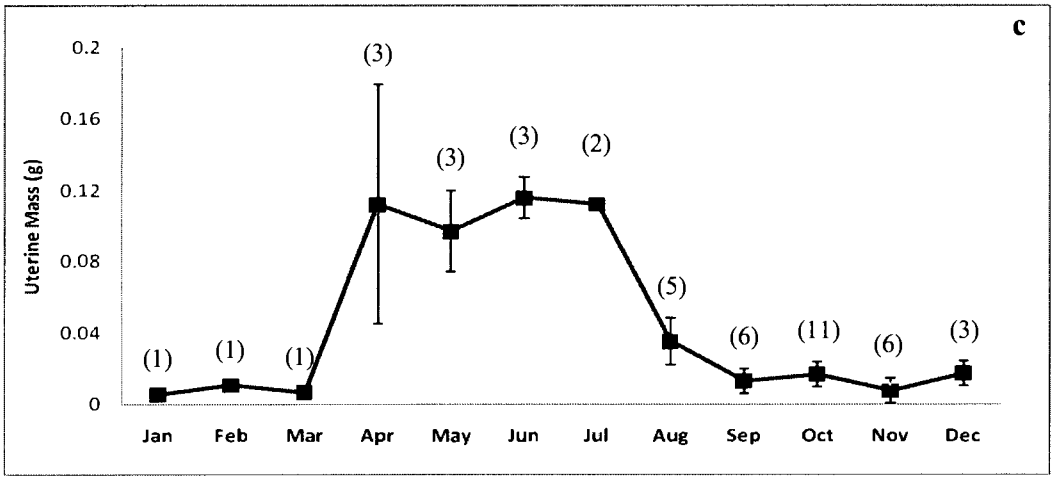


Figure 3.1 Continued…

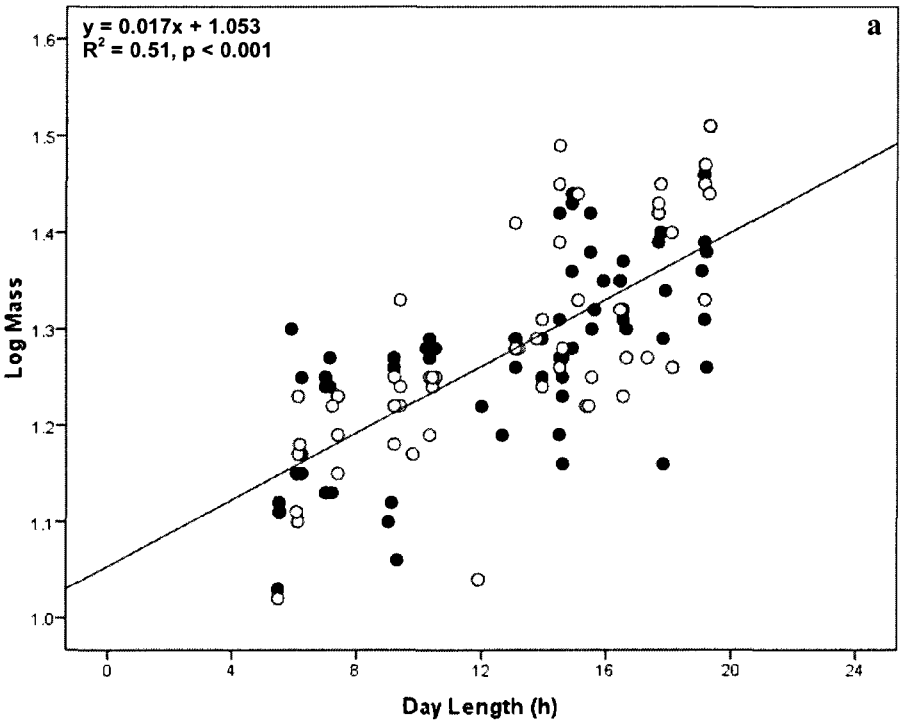


Figure 3.2 The Relationship Between Photoperiod, Body Mass, and Body Fat in Free-Living Northern Red-backed Voles (*Myodes rutilus*) Trapped Between November 2004 and August 2006. (*a*) Body Mass vs. Day Length. Shaded circles correspond to males and open circles to females. Mass was directly proportional to day length and increased with increasing photoperiod ($y = 0.017x + 1.053$, $R^2 = 0.51$, $p < 0.001$) (*b*) Total Fat Mass vs. Day length. Total body fat was directly proportional to day length and increased with increasing photoperiod ($y = 0.010x + 0.163$, $R^2 = 0.15$, $p < 0.001$). (*c*) Percentage Body Fat vs. Day length. Percentage body fat did not vary seasonally ($y = -0.006 + 4.55$, $R^2 < 0.001$, $p = 0.753$).

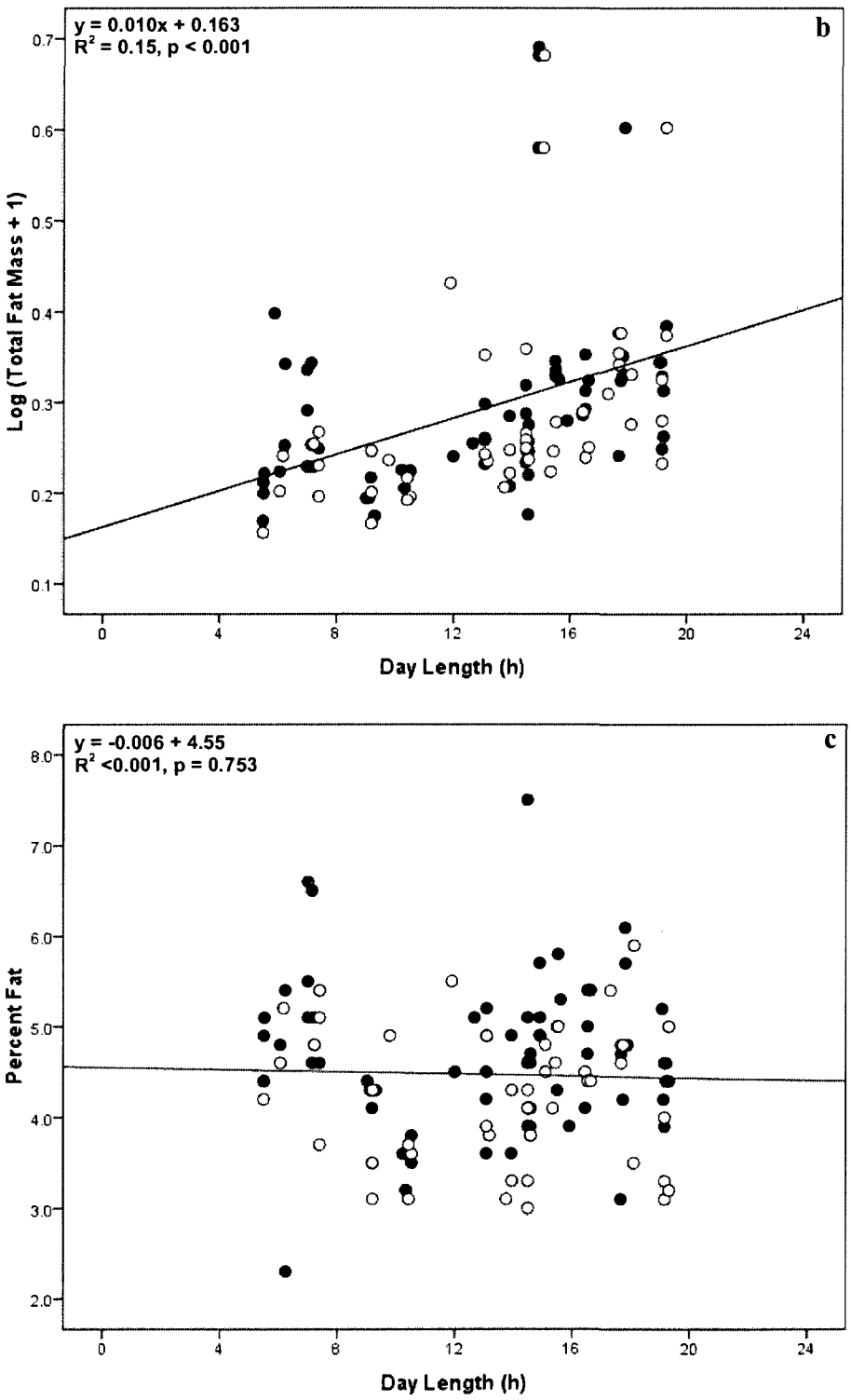


Figure 3.2 Continued…

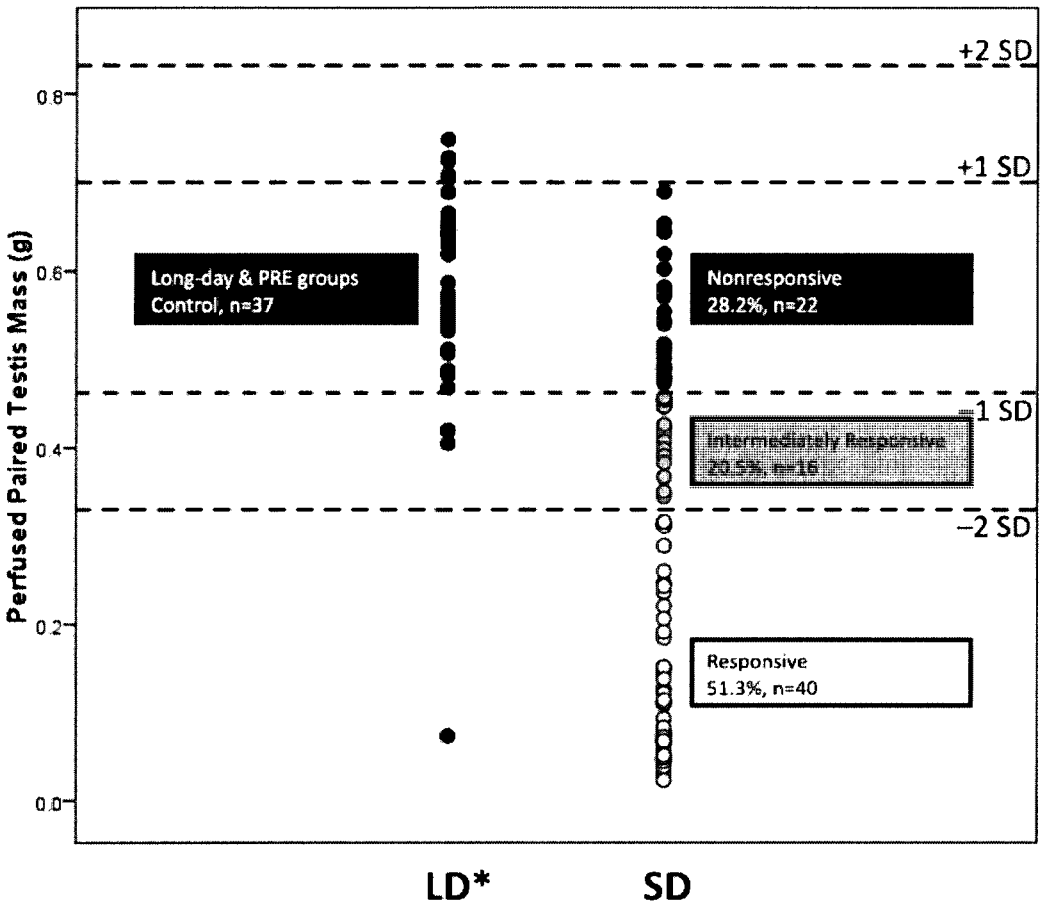


Figure 3.3 Intraspecific Variation in Reproductive Response to Short Photoperiod in an Arctic/Subarctic Arvicoline Rodent Species, the Northern Red-backed Vole (*Myodes rutilus*). Photoperiod non-responsive voles had perfused paired testis masses within 1standard deviation (s.d.) of the mean of LD* animals. The percentage of voles not responsive to photoperiod (28.2%, $n = 22$) fell within the 20-40% range known for lower latitude species. Intermediate responders (20.5%, $n = 16$) had paired testis masses between -1s.d. and -2s.d. of the mean of LD* animals, while photoperiod responsive voles (51.3%, $n = 40$) had testis masses at least 2s.d. below the LD* mean.

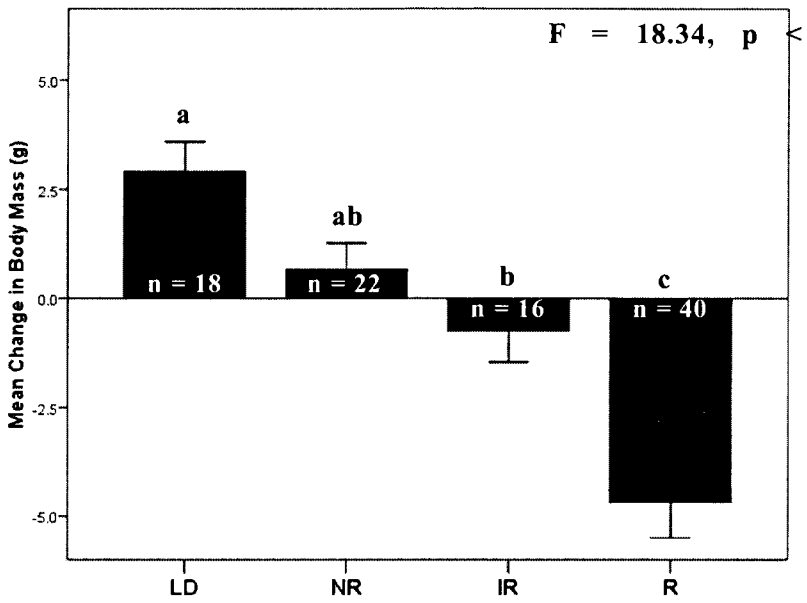


Figure 3.4 The Relationship Between Photoperiod, Body Mass, and Reproductive Grouping in Captive Northern Red-backed Voles (*Myodes rutilus*). Captive voles that remained on long days increased in mass while those switched from long to short days lost mass after 12 weeks. The overall change in body mass during the controlled study was significantly greater in the long day (LD) than in the short day groups (independent samples t-test, equal variances not assumed $p < 0.001$, not shown). Comparison of the overall change in body mass over 12 weeks for captive voles in four reproductive groupings: long day (LD), short day non-responsive (NR), short day intermediately responsive (IR), and short day responsive (R) (ANOVA, $F_{[3,95]} = 18.34$, $p < 0.001$). Voles in LD and NR groups both maintained large testes and gained body mass. Intermediately responsive and responsive voles reduced in body mass. The body size of voles appears to change in proportion to testis size.

Chapter 4:
Differential Regulation of the Hypothalamic-Pituitary-Gonadal (H-P-G) Axis in Photoperiod Responsive and Non-responsive Red-backed Voles (*Myodes rutilus*)

4.1. Abstract

Arvicoline rodents exhibit a broad range of intraspecies variation in their reproductive response to short photoperiods, ranging from very responsive to non-responsive. In high-latitude regions, this has important implications for seasonal and out-of-season breeding, as well as population cycling. The mechanisms that underpin photoperiod non-responsiveness have not been fully identified, but are likely to be detected along the hypothalamic-pituitary-gonadal (H-P-G) axis. Captive male northern red-backed voles (N = 96) were housed under long days (18L:6D) and age-matched into two groups. One group was switched to short days (8L:16D, n = 78), while the other (control) remained on long days (n = 18) for twelve weeks. Voles exhibited a highly variable phenotypic reproductive response to short dayength and were grouped as either non-responsive (NR, n = 22), intermediately responsive (IR, n = 16), or responsive (R, n = 40). I used immunohistochemical techniques to stain and quantify two hypothalamic neurons responsible for affecting reproduction, gonadotropin-releasing hormone (GnRH) and gonadotropin-inhibitory hormone (GnIH). Radioimmunoassay was used to quantify levels of luteinizing hormone (LH) and testosterone. Testosterone was significantly different between groups (ANOVA ($F_{[3,100]} = 35.61$, $p < 0.001$). There was no significant difference in LH between groups, but LH did show a pattern similar to that observed in testosterone in that it was highest in LD*/NR, next highest in NR/IR, and lowest in IR/R). LH and testosterone were also both significantly correlated with testis mass (Pearson correlation, LH n = 47, r = 0.302, p = 0.039, Testosterone n = 104, r = 0.602, $p < 0.001$). GnRH staining was

successful, but only partial staining of GnIH neurons was observed, even despite implementation of several different antigen retrieval and amplification methods. Mean and median GnRH cell count did not vary among groups (mean: ANOVA $p > 0.05$; median: Kruskal-Wallis $p > 0.05$), but intraspecies differences could still exist among GnIH neurons, GnRH cell area and optical density, or in other biochemical factors present at the hypothalamic level. Taken together, these results suggest that the phenotypic variation observed in red-backed voles occurs at or above the pituitary level.

4.2 Introduction

Arvicoline rodents (voles and lemmings, subfamily *Arvicolinae*) in arctic and subarctic regions often have pronounced multiannual population cycles and are a major food source for many predatory birds and mammals (Wilson and Bromley, 2001; Gilg *et al.*, 2003; Hudson and Bjornstad, 2003). These animals are also important to many secondary prey species that can suffer high rates of predation during low vole or lemming years (Wilson and Bromley, 2001). Breeding and reproductive timing in arvicoline rodents are, therefore, key elements of predator-prey dynamics in northern ecosystems.

Most high-latitude voles and lemmings are summer breeders, but rare and variable occurrences of winter breeding have been documented in almost every species studied, including the northern red-backed vole (*Myodes rutilus*) (Khlebnikov, 1970; Whitney, 1976; Hansson, 1984; Millar, 2001). Out-of-season breeding can occur only in certain individuals termed 'photoperiod non-responsive morphs' which do not regress their testes in response to short daylengths, although they do show other responsiveness. Typically, 20-40% of voles in captive small rodent populations possess this ability, but the percentage of animals expressing this phenotype in captive colonies decreases under cold ambient temperature (and possibly caloric) challenge (Nelson, 1987; Kriegsfeld *et al.*, 2000a; Stevenson *et al.*, 2009).

Photoperiod affects the production of melatonin in the pineal gland of endotherms, which in turn affects the production of two hypothalamic neuropeptides, gonadotropin releasing hormone (GnRH) and gonadotropin inhibitory hormone (GnIH) (Roy and Belsham, 2002; Ubuka *et al.*, 2005; Tsutsui, 2005). These hormones are produced in specialized cells in the pre-optic area (POA) and dorsomedial hypothalamus (DMH) of the brain, respectively (Kriegsfeld *et al.*, 2006, Bentley *et al.*, 2006). GnRH stimulates reproductive processes by influencing production of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in the pituitary. These gonadotropins stimulate and/or maintain testosterone production, testicular growth, and spermatogenesis. Conversely, GnIH inhibits reproductive function by acting upon both GnRH in the POA and the gonadotropins in the pituitary (Tsutsui, 2005). The majority of voles and lemmings are photoperiod responsive, and their testes regress under short photoperiods. In every species studied, however, some individuals do not respond to short photoperiods and maintain large testes (Nelson, 1987; Kriegsfeld *et al.*, 2000a; Stevenson *et al.*, 2009).

The hypothalamic-pituitary-gonad (H-P-G) axis of small mammals is upregulated during long daylengths and downregulated during short daylengths (Wallen and Schneider, 2000). Upregulation of the axis begins at the hypothalamic level within cell bodies of GnRH-secreting neurons. GnRH is shuttled from these cell bodies along beaded fibers stretching through the median eminence to the pituitary, where it acts to stimulate gonadotropin production. Luteinizing hormone (LH) is secreted at the pituitary level and transported through the blood to Leydig cells in the male gonads (testes), which contain LH receptors. Leydig cells produce testosterone upon stimulation by LH. Testosterone and follicle stimulating hormone (FSH) are important for gametogenesis, which occurs among Sertoli cells of the testes (Holstein *et al.*, 2003). Downregulation of the H-P-G axis typically occurs through inhibitory affects of short day lengths on GnRH production (Wallen and Schneider, 2000).

The hormonal cascade along the axis quickly terminates, and gametogenesis is halted in the absence of necessary hormonal inputs. The mammal subsequently undergoes gonadal regression. However, non-responsive morphs (animals that do not regress their testes in response to short daylengths) occur within most small mammal populations (Nelson, 1987; Kriegsfeld *et al.*, 2000a), although the mechanism is not known.

The northern red-backed vole exhibits a change in body mass that is positively correlated with gonadal mass, although the link between body mass change and reproductive phenotype under short day exposure remains unclear (Stevenson *et al.*, 2009). Age could also be related to the expression of the non-responsive phenotype, since animals that live longer might be able to produce more viable offspring that survive and reproduce. Since the average life span of free-living arvicoline rodents is short (<1 year) (Bronson 1989), an older animal might benefit by continuing to breed rather than risk overwintering in regressed condition and taking the chance that it would survive to upregegulate its reproductive axis again the following spring. Additionally, changes in daylength and reproductive condition can also affect immune function in small mammals (Demas and Nelson, 1998). Presumably, there is a trade off between the energy that is invested into reproduction and that which is invested into immune function. Photoperiod alone may help these animals to predict the time when a strong immune system is most needed and might, by itself, induce immune changes. Additionaly, immune function declines sharply upon interaction with moderately cold temperature (perhaps another predictor) (Demas and Nelson, 1998). Analysis of splenic mass is not the best way to understand the immune system, but it has been linked to immune function in small mammals (Prendergast and Nelson, 2001; Corbin *et al.*, 2008). The mating system of arvicoline rodents may influence endocrine-immune interactions, and the suppressive effect of testosterone on immune function may be why males have lower immune responses than females (Klein and Nelson, 1998).

The physiological mechanisms underpinning photoperiod non-responsiveness in voles and lemmings are not well known (Kriegsfeld *et al.*, 2000a). As non-responsiveness could be linked to differences in hormonal regulation at the hypothalamic level, establishing methods of quantifying neurons that regulate the H-P-G axis in northern red-backed voles will be necessary. To test for differences in reproductive regulation along the H-P-G axis, a clear understanding of the position and location of GnRH and GnIH neurons in the study species is required. At present, no brain atlas is available for *M. rutilus*, and the stereotaxic location of cell bodies and fibers in the brains of this species is not known. There have also been no prior attempts to test differentiation at each level of the H-P-G axis (hypothalamus, pituitary, and gonads). Whether the genetic differences leading to the observed intraspecific phenotypic differences in small mammal reproductive systems (Nelson, 1987) act at the hypothalamic level, pituitary level, gonadal level, or elsewhere is unknown. My aim was to identify the position of GnRH and GnIH neurons in the hypothalamus of *M. rutilus* in stereotaxic coordinates for use in future comparative treatment studies, and to measure differences in hormone production at each level of the H-P-G axis.

4.3 Methods

4.3.1 Identification and Mapping of GnRH and GnIH Neurons

I collected the brains of two captive northern red-backed voles (one raised on long days (16L:8D), and one photoperiod responsive animal raised under short days (8L:16D). The voles' brains had been perfused with a saline wash solution and paraformaldehyde solution (details discussed below in 4.3.2) at the University of Alaska Fairbanks. The voles' brains were soaked overnight in parformaldehyde, transferred to a sucrose-based cryoprotectant solution, and embedded in gelatin. Using a cryostat, two full *M. rutilus* brains were sectioned into 40μm sections, beginning at the olfactory bulb and moving caudally. I used the immunohistochemical techniques described in Parry *et al.* (1997) to stain

every third section for GnRH and GnIH neurons. In general, sections were washed in buffer, incubated in blocking serum, incubated for 48h in primary antibody, incubated in universal secondary antibody, stained with chromogen, soaked in mounting solution, and mounted on slides. Once dry, slides were dehydrated by placing them in ethanol and xylene and coverslipped.

To test the specificity of each antibody, a preabsorption test was run with each peptide (GnRH, Sigma L7134; GnIH, donated from K. Tsutsui, Japan). Primary antibody solutions were prepared at working dilutions and containing saturating concentrations of GnIH (5ug/ml solution) or GnRH (1ug/ml solution). Representative brain sections were placed into solution and incubated normally. The lack of staining confirmed that the antibodies were specific. Positive controls were used in these tests (comparable sections from the same brains and brain region incubated in the saturated solution), which stained for GnIH and GnRH in respective solutions. An additional series of tests were run in which sections were treated normally, except that either the primary antibody (GnRH LR-1, chicken, donated by Pierre Deviche, Arizona State University; GnIH, quail, donated by K. Tsutsui, Japan), the secondary antibody (Vectastain ABC Kit PK-6200, Vector Labs), or the chromogen Vectastain SG Substrate Kit SK-4700, Vector Labs) was replaced with buffer. Again, the result of these tests (against positive controls) was no stain present, ensuring specificity at each level.

Using a microscope and a mouse brain atlas (Franklin and Paxinos, 2008), I mapped the location and position of GnIH and GnRH cell bodies and fibers. I used representative thionin-stained sections (every third section of the POA and DMH, sliced rostral to caudal) from each brain to recognize neuroanatomical structures and landmarks in that same brain. This allowed me to correctly match atlas figures to the proper slides and sections of vole brains. The vole and mouse brains were sufficiently similar, enabling me to trace the outlines of every other figure in the atlas from Figs. 15-61 and plot separate GnRH and GnIH schematics of cell bodies and fibers identified in the vole brains. The remaining two sections

(out of every three sections sliced) were stored in cryporotectant solution until immunostaining. One was analyzed, and one was kept as a reserve.

In general, GnRH cell bodies were much darker and more visible than GnIH cell bodies, which did not show complete staining. The GnIH antibody concentration was elevated to 1:5000, and again to 1:2500, but this did not produce a complete stain of the neurons. Only partial staining of some cell bodies and fibers were observed, but it is possible that little antigen was present. I performed several noted antigen retrieval methods following Shi *et al.* (1997), including combinations of increased temperature (60°C, 90°C) and emersion in citrate buffer (weak and strong molarity) for different lengths of time. These methods, however, did not substantially improve the staining profile for GnIH. I then attempted a GnIH amplification protocol that can enhance staining of cell bodies in mammals (0.1 x GnIH concentration + biotinylated tyramide incubation, L. Kriegsfeld, pers. comm.), but this was also unsuccessful. The staining of GnIH neurons was sufficient to generate a map of the position and distribution of stained cell bodies and fibers, but not for quantification of these neurons. This may be due either to a lack of sufficient antigen in the brains, low binding affinity of the vole antigen to the quail primary antibody, or to the staining method. After mapping cells in these two individuals, the number of GnRH-ir cells in the POA (mean per POA, median per section) were compared in voles from each group (Long Day (LD), Non-responsive (NR), responsive (R), n = 6 per group).

4.3.2 Captive Animal Experimentation

From 2006 to 2007 I bred and raised captive *M. rutilus* from a pre-existing captive northern red-backed vole colony in animal quarters at the University of Alaska Fairbanks campus (IACUC protocol #06-53). The colony was composed of animals originally trapped in Interior Alaska in 2000, and was diverse in terms of both age and ability to entrain to different circadian rhythms (Tavernier *et al.*, 2004). We selected a homogenous subset of captive male voles that had been

raised and maintained on long photoperiods (16L:8D). These voles were then age-matched (age range from 60 to 300 days) and categorized into three groups: Pre-study control ('PRE', 16L:8D, $n = 20$), continued long day treatment ('LD', 16L:8D, $n = 18$), and short day treatment ('SD', 8L:16D, $n = 78$). Thirty days prior to the study, male subjects were separated from the colony and placed in individual cages. LD and PRE cages were kept on racks in a photoperiod-controlled room, while SD cages were kept in photoperiod-controlled chambers (up to 12 animals per chamber). All subjects remained on long photoperiods at room temperature (20°C) during this time and were given *ad libitum* access to food and water.

Prior to the start of the study and while all animals were still on long days, I measured the external testis volume and body mass of each animal. I anesthetized voles using an isoflurane vaporizer interfaced with a plastic holding container and a re-breathe bag. I weighed each animal, shaved the left side of the scrotum, and obtained a left testis length and width (to the nearest 0.01 mm) using digital calipers. At the start of the experiment, all of the animals in the 'PRE' group were euthanized so that samples from them could be used for comparisons with the other groups at the end of the experiment, and as an additional control – especially with respect to the voles in the LD group. Voles were euthanized via an overdose of sodium pentobarbital (50 to 75mg/kg, IP). A toe-pinch reflex test was used to ensure that they were unconscious, enabling a blood draw by cardiac puncture while the heart was still beating. Voles then underwent thoracotomy and blood draw via cardiac puncture (left ventricle). After cutting the right atrium, voles were perfused through the right ventricle with a saline wash solution for two minutes (0.9% NaCl in 0.1M phosphate buffer, 0.1% $NaNO_2$) and a paraformaldehyde solution (4% paraformaldehyde in 0.1M PB, $NaNO_2$) for 8 minutes.

After perfusion, the testes, spleens, and brains were removed; and testes and spleens were weighed. I determined paired testis mass and actual testis volume of

each subject. I then began the 12-week photoperiod for the remaining two groups. The LD animals remained on long photoperiods, while the SD animals were switched to short photoperiods. All animals remained on *ad libitum* food and water and were kept at 20°C throughout the treatment. After 12 weeks, the LD and SD animals were euthanized in the same manner as the PRE animals.

Quantification of GnRH-ir cells occurred by counting cell bodies in the medial and lateral POA and multiplying by three, since every third section of the POA was stained and analyzed. Mean and median values were recorded for each section and for the entire POA. The median value was determined to be that value falling directly between all cell count values from lowest to highest. All procedures were approved by the UAF Institutional Animal Care and Use Committee (IACUC) committee (#06-53).

4.3.3 Hormone Assays

Slides stained for GnRH peptide were analyzed by counting the number of GnRH-ir cells in every third section of the POA. I then determined whether the mean number of GnRH-ir cells per brain (POA only) or the median number of GnRH-ir cells per brain section varied by group (n = 6).

Luteinizing hormone concentration [LH] was determined by radioimmunoassay (RIA) at IZOTOP Institute of Isotopes, Inc. (Budapest, Hungary) using specific [^{125}I] kits for rat LH with known cross-reactivity in several other rodent species. Plasma volumes obtained from cardiac punctures (collected immediately before the perfusion process) were insufficient to run both testosterone and LH in duplicate for most animals (at least100µl required per run, determined experimentally), so single runs were used for LH.

Testosterone concentration [T] was determined by RIA at the University of Alaska Fairbanks. Plasma samples were frozen at -80C until assay analysis. For each plasma sample (50µL per tube), I determined T concentration in duplicate

following extraction in dichloromethane using direct radioimmunoassay as detailed in Wingfield *et al.* (1992). Each plasma sample was equilibrated with 2000cpm of radiolabeled testosterone to assess percent recovery. Average testosterone recovery was 80.25%. All samples were analyzed in a single radioimmunoassay (assay sensitivity 1 pg/tube), and intra-assay variability was 2.96%.

4.3.4 Statistical Analyses

I used analysis of covariance (ANCOVA) to identify whether differences in reproductive phenotype were related to age or body mass, and I used a Pearson's correlation to determine whether testis mass, testosterone level, and LH level were related. I also used analysis of variance (ANOVA) with Tukey's HSD tests to determine whether differences in mean GnRH-ir cell count, LH level, testosterone level, or relative spleen mass varied among groups. A Kruskal-Wallis test with a correction factor for tied ranks was used to analyze data for median number of cells per brain section.

4.4 Results

A strong correlation existed between perfused testis mass and both perfused testis volume ($R^2 = 0.92$, $p<0.001$) and the estimated external testis volumes at week ten for LD and SD voles ($R^2 = 0.66$, $p<0.001$), ensuring that perfusion did not affect the integrity of the testis mass data. Based on the final paired testes mass of PRE and LD animals, the twelve additional weeks of long day photoperiod treatment did not cause LD animals to become photorefractory (a regression of the testes due to overexposure of long days) relative to PRE animals that were euthanized twelve weeks earlier (PRE 0.5656 ± 0.0139; LD 0.5952 ± 0.1479; t-test, $p = 0.941$). Only one LD animal had regressed testes, but this did not cause any significant difference between the groups. These two groups were,

therefore, combined into one large group ('LD*', $n = 37$) for testis mass comparisons with SD animals.

The means and standard deviations of LD* paired testis masses were calculated to determine the different photoperiodic morphs within SD animals (Nelson *et al.*, 1989; Kriegsfeld and Nelson, 1999). Any SD animal that had a perfused paired testis mass within 1 standard deviation of the LD* mean ($n = 38$, 0.5796 ± 0.0204) was categorized as a photoperiod non-responsive morph (NR $n = 22$, 0.5457 ± 0.0137). Those falling between 1 and 2 standard deviations below the LD* mean were classified as intermediately responsive morphs (IR $n = 16$, 0.4135 ± 0.0095), and those having a testis mass below 2 standard deviations of the LD* mean were classified as photoperiod responsive morphs (R, $n = 40$, 0.1397 ± 0.0145). Reproductive phenotype was related to body mass ($p < 0.001$), but not age ($p = 0.31$) (Table 4.1; Stevenson *et al.*, 2009).

4.4.1 GnRH and GnIH

In both the LD and SD vole brains studied, GnRH and GnIH neurons were found in positions and stereotaxic locations similar to the rat, mouse, and Syrian hamster (Figs. 4.1 and 4.2; Kriegsfeld *et al.*, 2006). GnRH cell bodies and fibers were abundant in the pre-optic area (POA) (Figs. 25-36 in Franklin and Paxinos (2008) mouse brain atlas; Figs. 4.1 and 4.3). A complex network of GnRH fibers was found both rostral and caudal of this location in *M.rutilus* (Figs. 15-24 and 37-55 in Franklin and Paxinos (2008), respectively). GnIH cell bodies and fibers were present in the DMH (Figs. 42-49 in Franklin and Paxinos (2008) mouse brain atlas; Figs. 4.2 and 4.3). Both mean GnRH cell count per POA and median GnRH cell count per brain section of the POA did not differ significantly ($p > 0.05$; Fig. 4.4).

4.4.2 LH, Testosterone, and Splenic Mass

LH did not differ significantly between groups (Fig. 4.5, N = 47, p = 0.464), but did show a trend that was similar to testosterone, which did differ significantly between groups ($F_{[3,100]} = 35.61$, $p<0.001$, Fig. 4.5). The LD group had the highest LH levels, NR and IR were second highest, and R was lowest. LH and testosterone were also significantly correlated to testis mass (Pearson correlation, LH n = 47, r = 0.302, p = 0.039, Testosterone n = 104, r = 0.602, $p < 0.001$). LH was only slightly less correlated with testosterone than with testis mass (n = 46, r = 0.287, p = 0.053). A two-way analysis of covariance (ANCOVA) showed no significant effect of age on relative splenic mass (p = 0.71), but there was a significant effect of experimental group ($F_{[4,109]} = 4.17$, p = 0.004, Fig. 4.6). Relative splenic mass was significantly different between PRE and LD group voles ($F_{[4,109]} = 4.17$, p = 0.004), but not among short day groups ($p > 0.05$, Fig. 4.6). Differences between long day treatment groups (Pre and LD) showed that splenic mass was elevated in response to prolonged exposure to long day lengths (+12 weeks, $p < 0.05$).

4.5 Discussion

I identified the position and stereotaxic location of GnRH and GnIH neurons throughout the brain of *M. rutilus*, a high-latitude vole species that is known to undergo photoperiod non-responsiveness and can breed in winter (Khlebnikov, 1970; Whitney, 1976; Hansson, 1984; Stevenson *et al.*, 2009). The location of these neurons in the northern red-backed vole suggests that GnIH has an inhibitory effect both upon the gonadotropins in the pituitary (as GnIH fibers stretch through the ME and presumably terminate near the pituitary) and upon GnRH in the POA because previous studies have found that GnIH neurons reaching forward into the POA come into direct contact with GnRH neurons, presumably inhibiting the production and/or release of GnRH (Bentley *et al.*,

2006). The large networks of GnRH cell bodies and fibers important for stimulating gonadotropin production are consistent with this animal's relatively fast transition into seasonal reproduction.

I observed complete staining of GnRH cell bodies and fibers, but only partial or very little staining of GnIH cell bodies and fibers in the same brains. The methods of perfusion and tissue preparation for vole GnIH were consistent with those for vole GnRH (which stained well), and avian GnIH (which also stained well) previously analyzed in the same lab using the same protocol. Most (but not all) vole GnIH cell bodies were very faint. Yet, they could be made more visible by altering the microscope light settings and focus beyond the normal settings for GnRH. Increased GnIH antibody concentration did not improve staining.

Antigen retrieval methods that are often used to relax proteins that may have changed slightly in their conformation, binding capacity, and specificity during the perfusion process (Shi *et al.*, 1997) were unsuccessful in improving staining of GnIH, as were other amplification methods (see Methods). The partial GnIH staining, therefore, could be due to either low binding affinity of the vole antigen to the quail antibody or to a much higher abundance of different type of GnIH peptide in the vole. Japanese quail GnIH antibody has been successfully used to stain for GnIH peptide in mice, rats, and Syrian hamsters (Kriegsfeld *et al.*, 2006), but with significantly reduced homology compared to other bird species. The GnIH peptide and antibody have yet to be purified, sequenced, or utilized in arvicoline rodents. Conversely, the GnRH antibody has been used extensively in mammals (Bronson, 1989), including arvicoline rodents (*e.g.*, prairie vole, *Microtus Ochrogaster* (Kriegsfeld *et al.*, 2000a). If the percent homology of the GnIH peptide in *M. rutilus* is decreased, even slightly, in comparison to that of the other rodent species that have been studied (which already show homology of < 50% to Japanese quail GnIH, Kriegsfeld *et al.*, 2006), this could explain the partial staining of GnIH cell bodies and fibers. Sequencing the GnIH peptide and

comparing the sequence with known sequences of avian and other mammalian peptide should determine this with certainty.

Alternate explanations for these findings are also possible. First is the possibility that the GnIH staining was complete and a true reflection of GnIH in the voles (*i.e.*, they simply don't generate a lot of the hormone). Another possibility is that the GnIH peptide was being shuttled very quickly down to the fibers, and did not remain in the cell body. A final alternate explanation for what was observed is that the peptide is blocked by another agent that could be removed by administration of pharmaceuticals.

My experimental groups did not differ in terms of the mean number of GnRH-ir cells per POA of the brain ($p > 0.05$), nor did they differ in median number of GnRH-ir cells per section of POA ($p > 0.05$). However, this does not necessarily mean that the mechanism underpinning non-responsiveness is not occurring at the hypothalamic level. Although some intraspecific differences in hypothalamic immunoreactive cell counts have been found in other arvicoline species (Kriegsfeld *et al.*, 2000a; Kriegsfeld *et al.*, 2000b), cell area and optical density can also vary significantly in arvicoline rodents (Kriegsfeld *et al.*, 2000a) other endotherms (Bentley *et al.*, 2003; Bentley *et al.* 2006). There may, therefore, be a difference at the hypothalamic level, just not one detected by measuring the mean or median number of GnRH-ir cells. If such a difference does exist, a measurement of cell area or optical density might more clearly detect a pattern correlated with testis mass among groups. Differences in GnRH fiber density or GnIH abundance may also contribute to GnRH release.

The H-P-G axes of the different photoperiodic morphs found in arvicoline species have not been well characterized, especially in high-latitude regions. There is, however, some evidence that non-responsiveness in lower-latitude arvicoline rodents is regulated at the hypothalamic level, and temperature is likely to have an effect (Kriegsfeld *et al.*, 2000a), not to mention effects of diet/nutrition on the H-P-G axis, which remains unstudied. The possibility that arvicoline non-

responsiveness is differentially regulated above the hypothalamic level has been investigated in *Peromyscus*, an unrelated rodent. Non-responsive morphs do not vary from responsive morphs in their pineal melatonin content, melatonin secretion patterns, brain melatonin receptor numbers, or melatonin receptor binding affinity in this genus (Blank *et al.*, 1988; Carlson *et al.*, 1989; Weaver *et al.*, 1990; Heideman and Bronson, 1992). It seems unlikely, therefore, that arvicoline rodents would vary in these characteristics. Still, the possibility remains, especially since certain arvicoline species (*e.g.*, northern red-backed voles, *M. rutilus*; brown lemmings, *Lemmus sibiricus*; and collared lemmings, *Dicrostonyx groenlandicus*) inhabit higher latitude areas than *Peromyscus* and their reproduction is presumably more strongly tied to photoperiod (Bronson, 1989). If arvicoline rodents follow a similar pattern to that of *Peromyscus*, then non-responsiveness should be regulated downstream from pineal-generated melatonin and melatonin receptors on hypothalamic neurons. The mechanism driving non-responsiveness would, therefore, be related to intraspecific differences in genetic regulation of one or more of GnRH/GnIH, LH/FSH, or testosterone/estrogen synthesis, secretion, and/or receptor density.

The lack of significant difference in mean LH concentration between groups is likely to be the result of a lower sample size relative to testosterone. Mean values were highest in LD* animals and lowest in R animals for both groups, and LH and T were both positively correlated with testis mass ($p < 0.05$). These results, however, are independent of negative feedback occurring along the reproductive axis. The hypothalamic and pituitary levels of the H-P-G axis could, however, be studied independently of negative feedback via castration and/or hypophysectomy and subsequent measurement of GnRH-ir and LH levels. Regardless, the observed trend in LH was still similar to that which was observed in testosterone. Concentrations of both LH and testosterone were both significantly correlated with testis mass, and LH was only slightly less correlated with testosterone than with testis mass. The similar trends in group LH and

testosterone values (Fig. 4.5) and the significant correlations between gonadal mass and both LH and testosterone suggest that the intraspecific phenotypic variation in voles occurs at or above the pituitary level and is most likely to be related to differential variation in GnRH (*e.g.*, cell area or optical density), GnIH, or LH synthesis, release, and/or receptor density.

4.6 Conclusions

The locations of GnRH and GnIH fibers in the brain of the northern red-backed vole (*M. rutilus*) were similar to their locations in mammalian taxa. Now that they are identified and mapped, future studies that quantify GnRH and further improve the method of staining for GnIH in arvicoline rodents will be easier to carry out. The GnIH fibers in *M. rutilus* reach forward to the POA and are likely to be in direct contact with GnRH neurons (although this should be confirmed in this species), while the GnRH fibers form extensive networks throughout the hypothalamus. Fibers from both neurons stretched towards the ME and pituitary. Both neuropeptides may, therefore, be involved in regulating seasonal and/or out of season breeding in this species. GnRH-ir cell counts did not differ significantly between groups, but mechanisms of differential responsivity may still be occurring, and if so, are likely to manifest themselves through analysis of one or more of GnRH cell area, optical density, or fiber density. Mean values of plasma LH concentration did not significantly differ between groups, possibly due to effects of negative feedback from testosterone. The mean concentrations did, however, show a trend similar to that of plasma testosterone and mean testis between among groups. Concentrations of plasma LH and testosterone were significantly correlated with testis mass. Taken together, these findings suggest that differential regulation of the H-P-G axis by responsive and non-responsive morphs is still most likely to occur at or above the pituitary level.

4.7 Acknowledgements

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4.9 Tables

Table 4.1 Body mass, not age, is related to reproductive phenotype in captive northern red-backed voles (*Myodes rutilus*). Analysis of co-variance (ANCOVA) was used, with final body mass and age (days old) as possible co-variates. Final body mass was more strongly related to gonadal mass than age, which was not a significant co-variate. Age (days old) was also not a significant co-variate of relative testis mass ($p = 0.26$, not shown). Goups were defined as pre-study and long day (LD*), non-responsive (NR), intermediately responsive (IR), and responsive (R).

Dependent Variable	*df*	F	p-value
Corrected Model	5	114.2	<0.001
Intercept	1	20.6	<0.001
Final Body Mass	1	9.0	<0.001
Age	1	1.1	0.31
Group (LD*, NR, IR, R)	3	110.0	<0.001
Error	110		
Total	116		

4.10 Figures

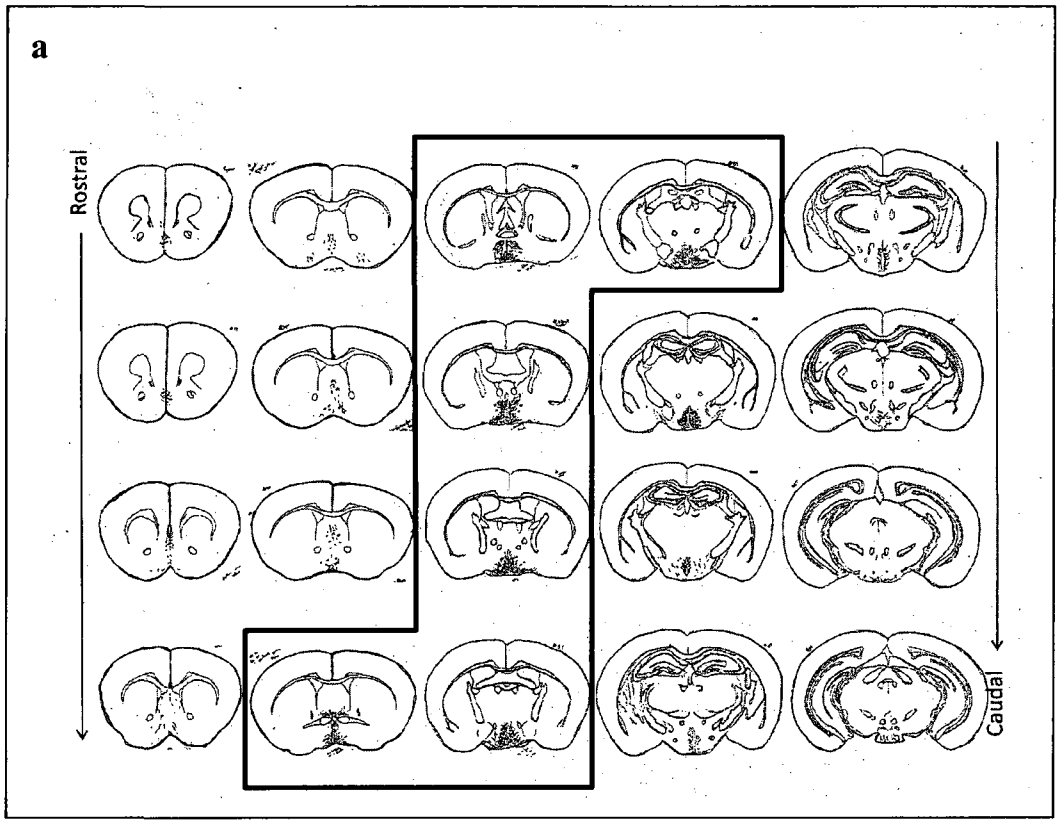

Fig. 4.1 Gonadotropin Releasing Hormone (LR-1) Cell Body and Fiber Distribution in the Northern Red-backed Vole (*Myodes rutilus*). Position of GnRH neurons in the hypothalamus of *M. rutilus* were plotted over corresponding tracings of mouse atlas figures (sections modified from Franklin and Paxinos, 2008; shown are Figs. 15-53, odd numbered only). Cell bodies and fibers were abundant in the pre-optic area (POA), highlighted here in Figs. 25-36 (sections modified from Franklin and Paxinos, 2008). A complex network of GnRH fibers extended rostrally and caudally from this region, as well as towards the median eminence and pituitary. Cell body and fiber distributions are shown in drawings of *a*) broad view of whole brain sections, and *b*) close view of the 3rd ventricle, oriented to lower right portion of each figure.

Figure 4.1 Continued…

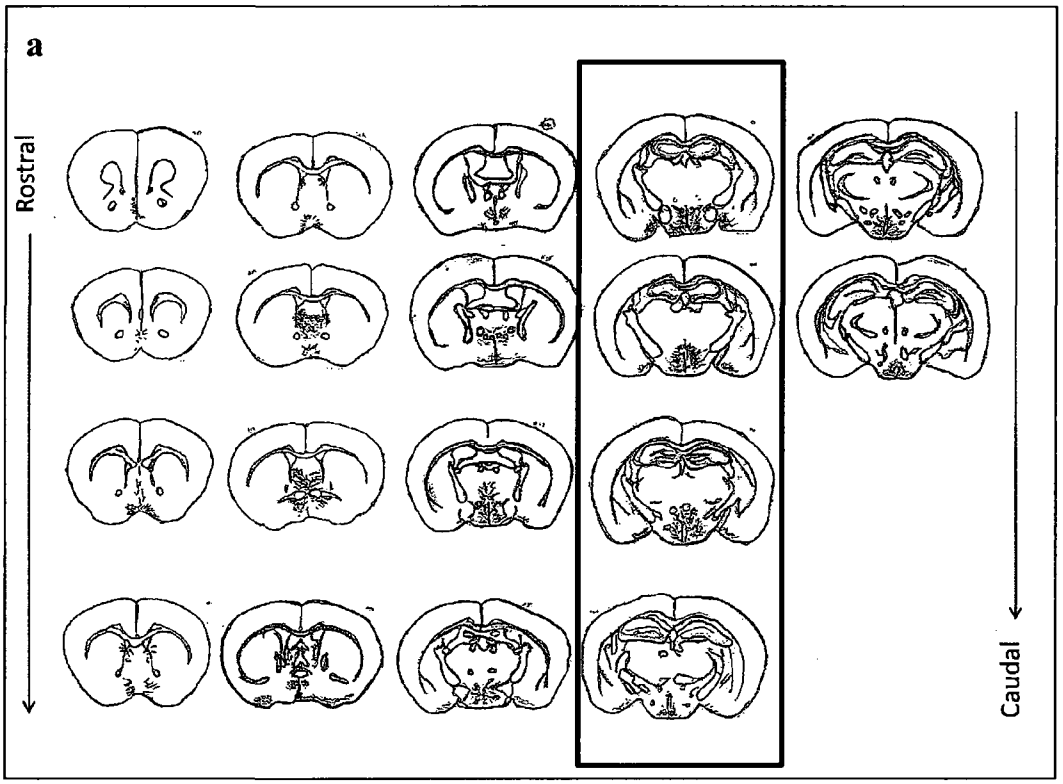

Fig. 4.2 Gonadotropin Inhibitory Hormone (GnIH) Cell Body and Fiber Distribution in the Northern Red-backed Vole (*Myodes rutilus*). Position and location of GnIH neurons in the hypothalamus of *M. rutilus* were plotted over corresponding tracings of mouse atlas figures (sections modified from Franklin and Paxinos, 2008; shown are Figs. 17-51, odd numbered only). Cell bodies and fibers were abundant in the dorsomedial hypothalamus (DMH), highlighted here in Figs. 42-49 (sections modified from Franklin and Paxinos, 2008). GnIH fibers extended ventrally towards the median eminence (ME) and pituitary, rostrally toward GnRH cell bodies in the POA, and caudally towards the spinal cord. Cell body and fiber distributions are shown in drawings of *a*) broad view of whole brain sections, and *b*) close view of the 3rd ventricle, oriented to lower right portion of each figure.

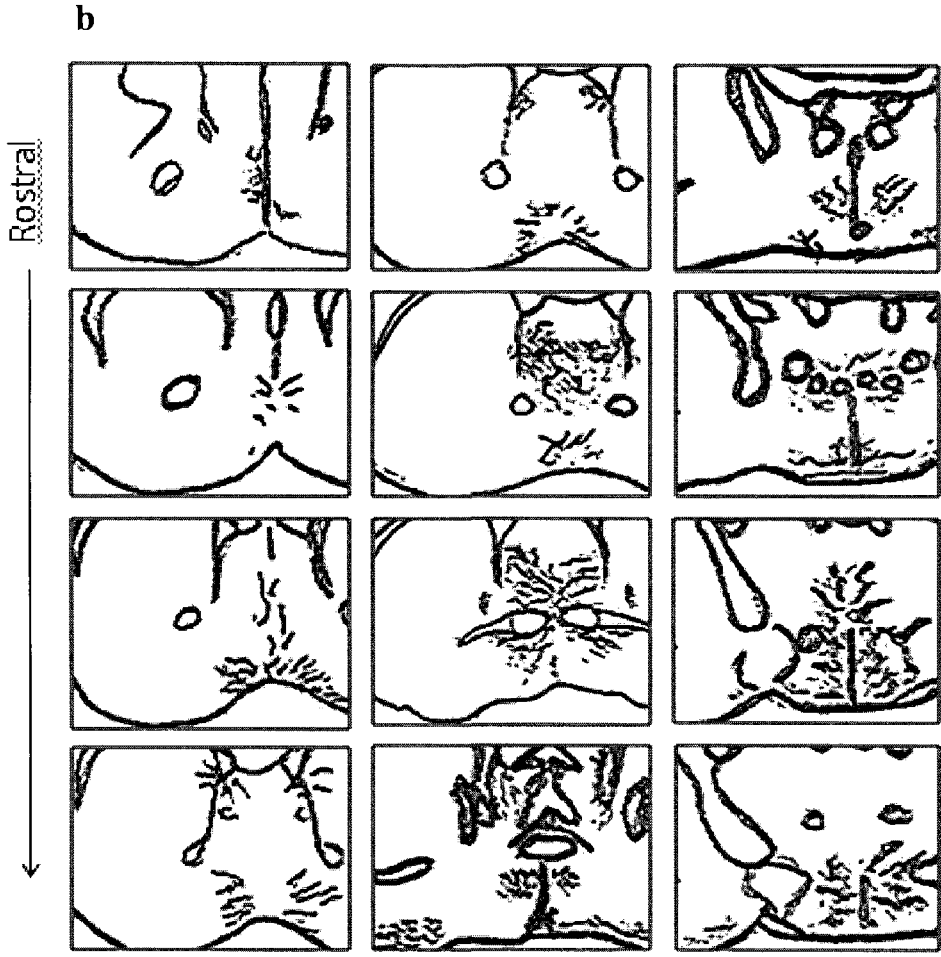

Figure 4.2 Continued…

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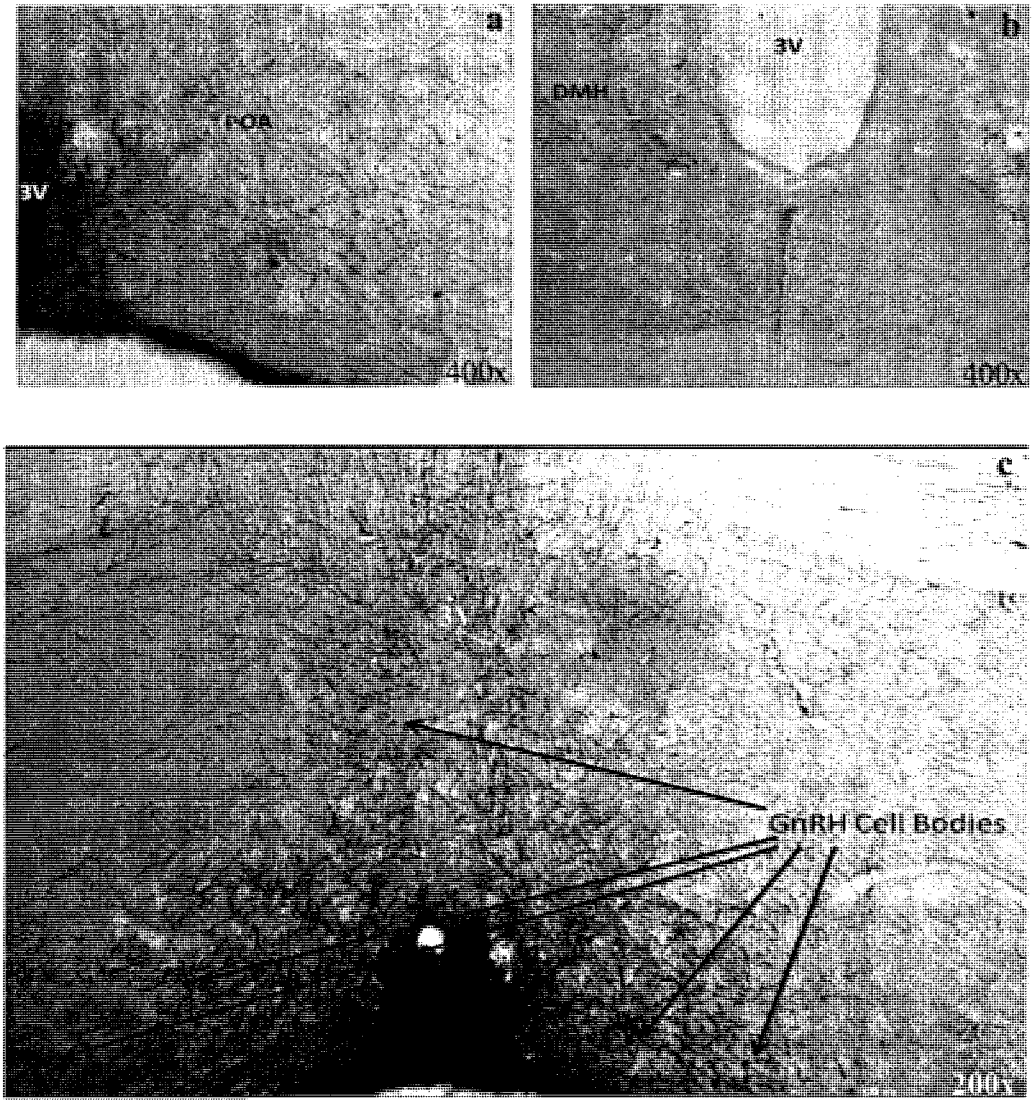
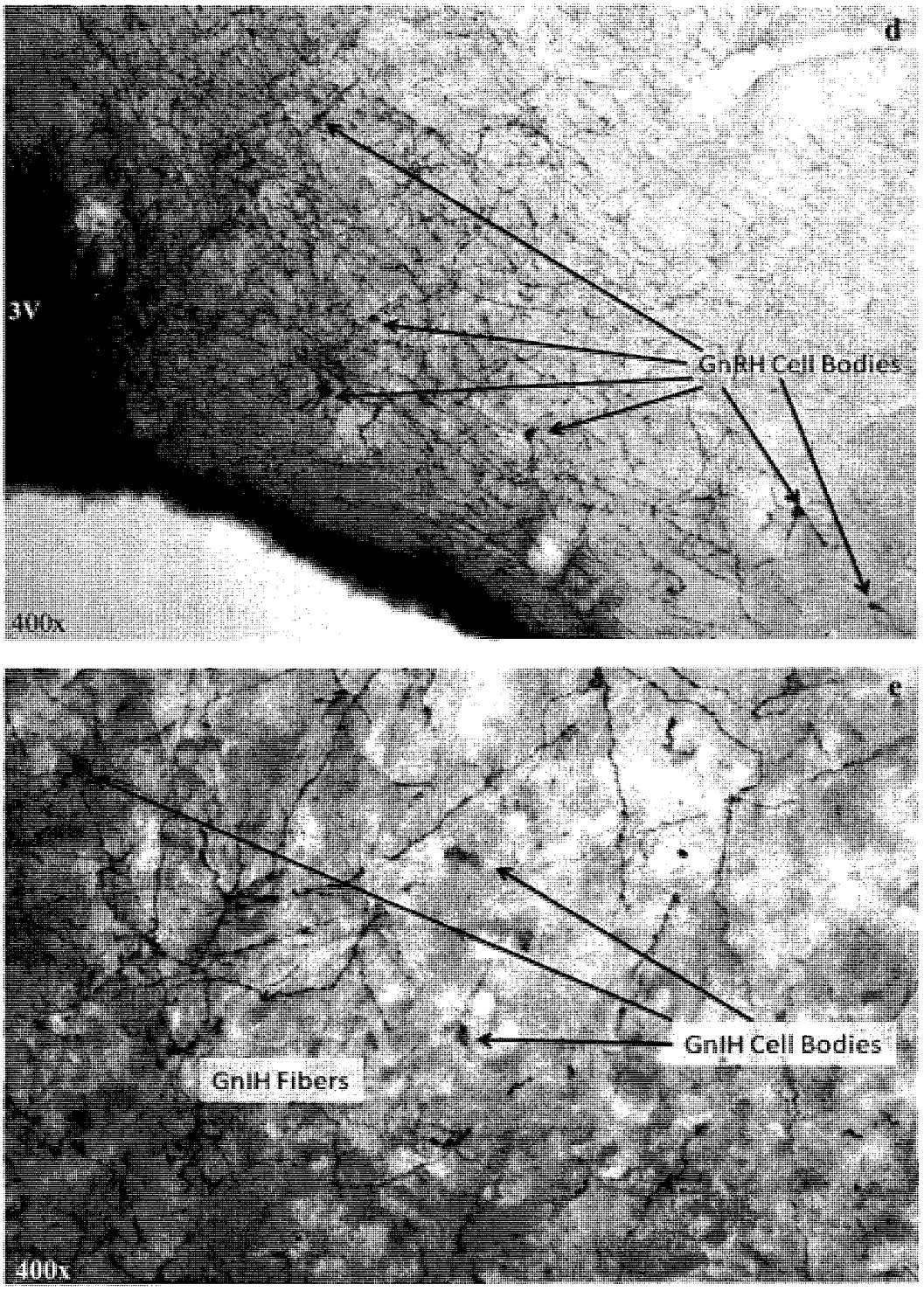


Figure 4.3 Effectiveness of Hypothalamic Staining for Immunoreactive Gonadotropin Releasing Hormone (GnRH-ir) cells in the Pre-Optic Area (POA) and Gonadotropin Inhibiting Hormone (GnIH-ir) cells in the Dorsomedial Hypothalamus (DMH) in brains of Northern Red-backed Voles (*Myodes rutilus*). Cell bodies and fibers are shown around the third ventricle (3V). Variation in cell and fiber abundance and staining quality were evident under normal contrast for *a*) GnRH in the POA and *b*) GnIH in the DMH. GnRH was suffucient for analysis under normal contrast lighting (*a*), but staining became more pronounced under high-contrast lighting (*c* and *d*). GnIH-ir cells were barely noticeable under normal contrast lighting (*b*), but became more visually apparent under high-contrast lighting (*e* and *f*). Unlike GnRH, GnIH-ir cells were not abundant, stained with poor quality, and could not be quantitatively analyzed.



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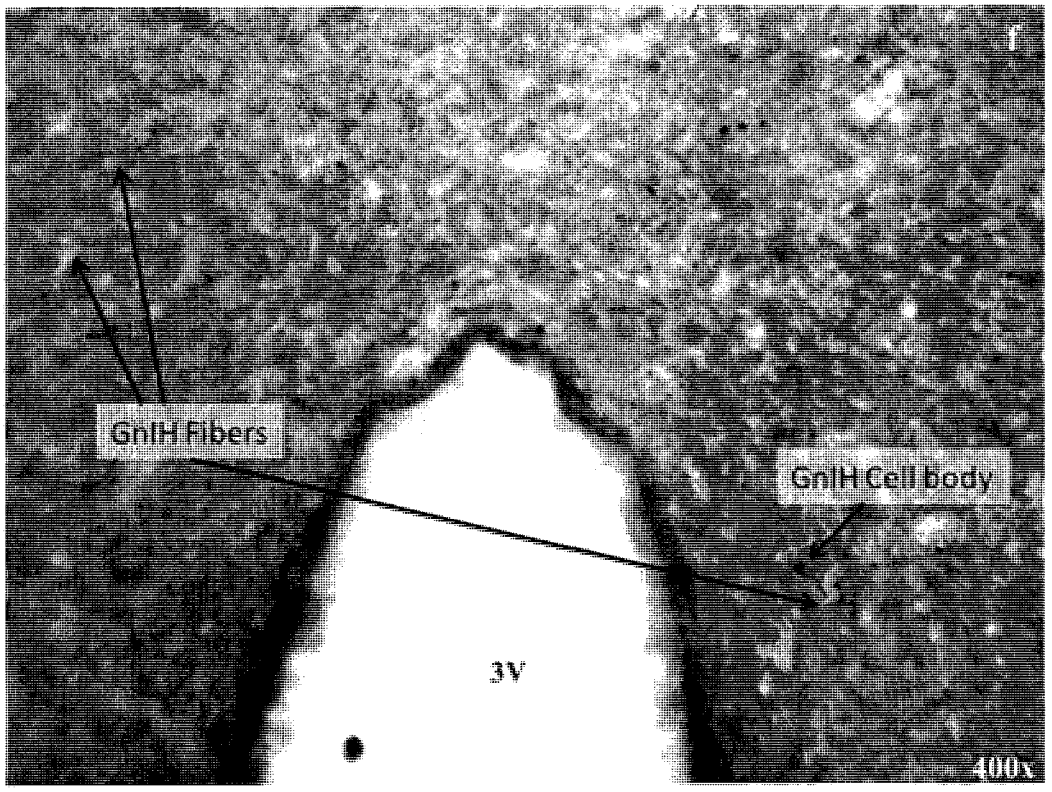

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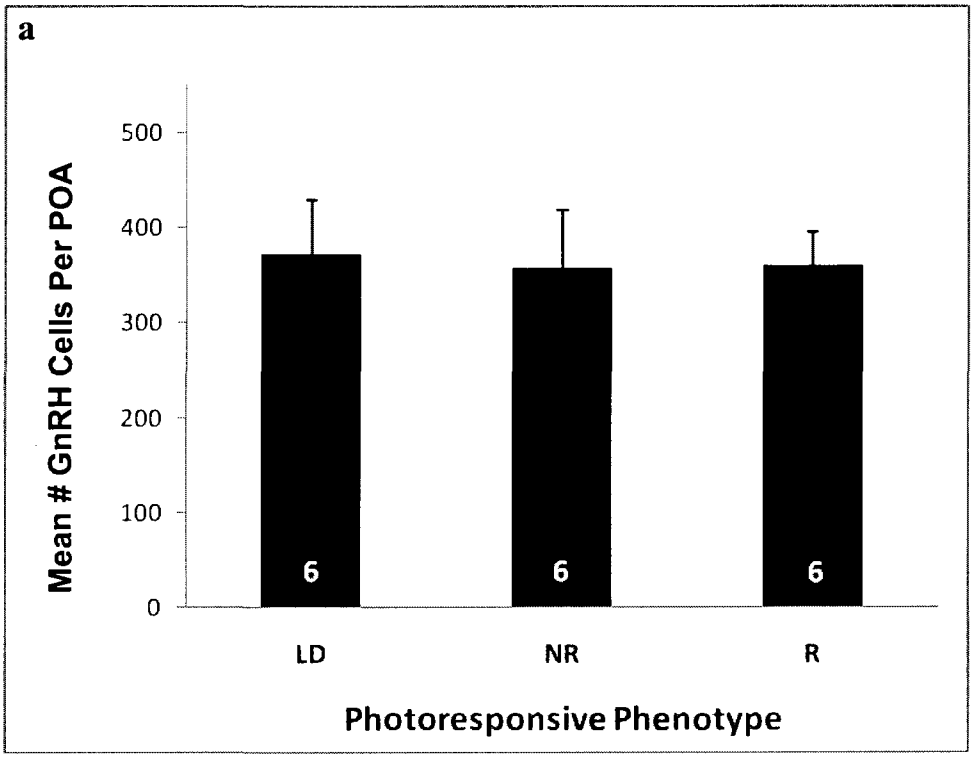
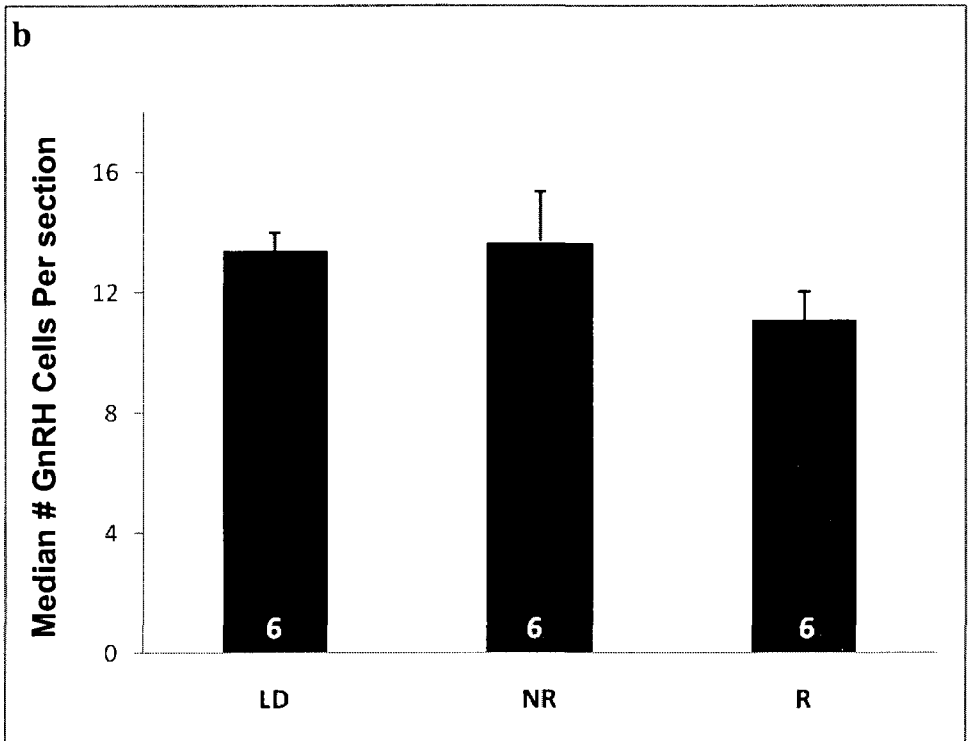


Figure 4.4 Cell Counts of GnRH Neurons Do Not Vary Among Different Phenotypes of Northern Red-backed Voles (*Myodes rutilus*). Long day (LD), non-responsive (NR), and responsive (R) voles were compared. Means and SEs are shown. (*a*) Mean number of GnRH cells per brain section in the pre-optic area (POA) (ANOVA, $p > 0.05$), (*b*) Median number of GnRH cells per brain section of POA (Kruskal-Wallis, $p > 0.05$).

Figure 4.4 Continued…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Median # GnRH Cells Per section
16
12
8
4
0
6
6
6
LD
NR
R

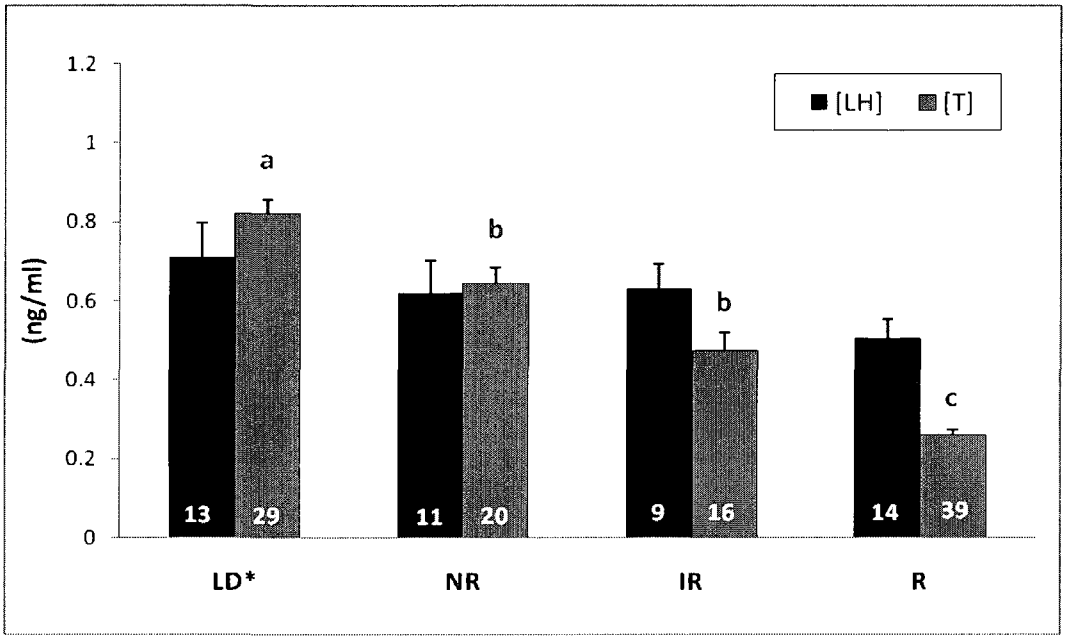

Figure 4.5 Intraspecific Production of Pituitary and Gonadal Hormones in Northern Red-backed Voles (*Myodes rutilus*). Analysis of Variance (ANOVA) showing differences among reproductive phenotypes. groups. Mean values and SEs are shown. Mean values represented by bars in the same color that share the same letter are not significantly different ($p > 0.05$). Testosterone was significantly different among groups ($F_{[3,100]} = 35.61$, $p < 0.001$). Although there was no significant difference in LH between groups, LH showed a pattern similar to that observed in testosterone in that it was highest in LD*/NR, next highest in NR/IR, and lowest in IR/R). Both hormones were also significantly correlated with testis mass (see results).

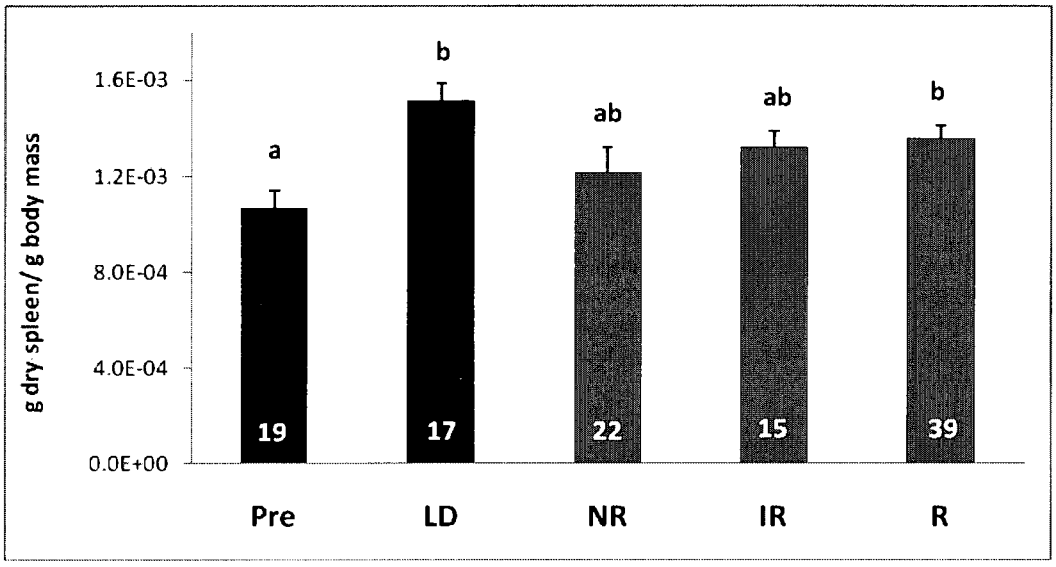

Figure 4.6 Relative Wet Splenic Mass Increases Following Prolonged Exposure to Long Daylengths, but Does Not Vary Significantly Between Short Day Reproductive Phenotypes. Means and SEs are shown. Mean values represented by bars that share the same letter are not significantly different ($p > 0.05$). Pre-study (Pre) animals and long day (LD) animals were control groups exposed to long day lengths (16L:8D, black bars).

Chapter 5:
Seasonality of Body Composition, Organ Mass, and Field Metabolic Rate in the Northern Red-backed Vole (*Myodes rutilus*)

5.1 Abstract

Small mammals in seasonal environments may allocate mass and energy towards or away from certain tissues, organs, or processes in different seasons. This reflects the temporary importance or lack of importance of a given component. The aim of this study was to measure the effect of season and several environmental parameters on body composition, visceral organ mass, and field metabolic rate (FMR) of free-living northern red-backed voles (*Myodes rutilus*) over several 12-month periods. I tested whether values differed by season or were correlated with proximate (environmental) factors. Notably, voles had larger protein reserves and relative dry masses (dry organ mass per g body mass) of liver and spleen in winter, while the relative size of mineral, heart, and stomach mass decreased. In summer, percentage protein, liver, spleen, and small intestine length increased. Cecum and stomach values were highest in spring and may be reflective of increased food intake at that time. The percentage of fat and the relative mass of the kidneys and intestines did not change seasonally. Body condition was most strongly related to photoperiod, while organ masses were strongly tied to gender and ambient temperature (T_a) ($p < 0.05$). Snow depth had a significant effect on most parameters in winter, but its overall contribution to the overall unexplained variance was minor (<10%). The FMR of voles did not change seasonally with respect to breeding season or sex (ANCOVA, fixed factors = breeding season and sex, covariate = T_a, $F_{[3,10]} = 2.465$, Corrected Model $p = 0.155p = 0.155$), but was correlated with T_a when sex and breeding season were combined ($R^2 = 0.29$, $p = 0.037$). Taken together, these results suggest that changes in climate could have a substantial physiological effect on *M. rutilus* and

alter the way in which energy is allocated.

5.2 Introduction

Seasons are predictable annual periods of time characterized by a variety of proximate factors, including temperature, snow cover, day length, precipitation, humidity, and others. Seasonal breeding becomes increasingly common and pronounced in rodents with increasing latitude, which is inversely related to the length of the breeding season (Bronson, 1989). In small mammals that inhabit highly seasonal environments, energy is spent on reproduction in spring and summer when it is not needed for thermoregulation to the same degree that it is needed in winter. Depending on environmental conditions, energy is partitioned either to maintenance (of which basal metabolic rate is a valid indicator), growth, activity, or reproduction (Speakman, 1997). These resource allocation requirements of animals are mutually exclusive and are thought to be largely independent of one other (Fisher, 1930). In other words, when available resources are limited, a small mammal is expected to exhibit an inverse relationship between the amount of energy that it allocates to reproduction and that which it allocates to growth and maintenance (Levins, 1968, Speakman, 1997). When energy is limited, the mass (which has an energy maintenance cost per gram) of certain anatomical regions, organs, or tissues may be decreased relative to others (Zuercher *et al.*, 1999; Song and Wang, 2006) to facilitate the fueling of thermoregulation or other seasonal processes.

A major route by which energy is diverted to maintenance is by the adjustment of metabolic rate. The principle of allocation predicts that resources available for reproduction should decrease as mass-specific basal metabolic rate (BMR) increases (reviewed in Speakman, 1997). For animals to expend large amounts of energy, they must have the alimentary capacity to process large amounts of food (Weiner, 1989; Hammond and Diamond, 1992; Wunder, 1993). They must, therefore, have larger or longer digestive tracts and organs. For a

given foraging strategy, the BMR, maximum food intake, and size of an animal's digestive tract and other organs are all correlated (Kirkwood, 1983).

Free-living small mammals inhabiting high-latitude environments undergo seasonal acclimatization that helps them to withstand extreme temperatures. They can, however, still be energy limited by food availability, thermoregulatory costs, or other factors. Additionally, food availability, physiological acclimatization, habitat use, movement patterns, and other behaviors all influence energy requirements (Bronson, 1989). The role of energetics in the patterns of resource allocation seen in small mammals is extremely complex and has not been fully resolved, but a clearer understanding of how small mammals partition their resources can help to identify which energy costs are associated with which allocation strategies. The aim of this study was to determine whether elements of seasonality were related to metrics of body composition, visceral organ masses, or the field metabolic rate (FMR) of northern red-backed voles (*Myodes rutilus*). By examining these seasonal patterns, I hoped to determine how or why voles allow changes in their physiology in response to season to facilitate either breeding or winter survival.

Arvicoline rodents (voles and lemmings) are small mammals that are common in the highly seasonal high-latitude environments of the Northern hemisphere. They neither migrate nor hibernate, and they are an important prey resource for many Northern predators (Wilson *et al.*, 2001; Gilg *et al.*, 2003). The northern red-backed vole in Alaska undergoes seasonal acclimatization in its physiology (Sealander, 1967; Sealander, 1972; Feist and Rosenmann, 1976; Feist, 1984; Zuercher, *et al.*, 1999; Stevenson *et al.*, 2009b) and behavior (Stebbins, 1974; West, 1977; Tavernier *et al.*, 2004), and must cope with problems of thermoregulation in different ways depending on environmental conditions. *Myodes rutilus* is also thought to undergo significant seasonal changes in a number of factors related to energy allocation and survival, including body composition (Whitney, 1976; Zuercher *et al.*, 1999), organ mass (Sealander 1967;

Zuercher et al 1999; Stevenson *et al.*, 2009b), and field metabolic rate (FMR) (Holleman *et al.*, 1982). To date, there has been no attempt to determine whether specific, abiotic environmental parameters can explain a substantial amount of the total variation observed in these physiological parameters in free-living *M. rutilus*.

Body fat percentages of arvicoline rodents may change seasonally in some populations (Anderson and Rauch, 1984), but not in others (Zuercher *et al.*, 1999). The lipid stores of arvicoline rodents are made up of a combination of white and brown fat which are utilized for thermogenesis in support of BMR costs. The metabolism of white fat cells provides a combination of stored chemical and heat energy, while brown fat, provides heat alone due to proton leak through uncoupling protein-1 (UCP-1) which decouples the proton motive force electron transport chain from oxidative phosphorylation (Criscuolo *et al.*, 2005). Lipids are also found in the form of free-fatty acids in the blood and in cell membranes. Taken together, a measure of total body lipid gives a general indication of energy reserves, insulation, and thermogenic capacity. I predict that the percentage of body fat will increase in winter, as the lean body mass of *M. rutilus* is thought to decrease in winter (Zuercher *et al.*, 1999), and winter in southcentral Alaska is relatively mild in terms of ambient temperature when compared to much of this species' range.

In this study, lean mass (LM) was defined as the sum of all fat-free, ash-free components. The first of these is body water (BW), which provides some indication of the hydration state of animals at capture. The category, "protein", is also a component of LM, is the sum of all organic compounds made up of amino acids that are joined by peptide bonds, and can be an important fuel for animals via gluconeogenesis. Mineral content refers to the sum of all minerals in the body that comprise the ash content. These components, along with residual lean mass items (*e.g.*, vitamins, DNA, RNA) constitute the overall LM of the animal.

Protein can serve as a carbon source for carbohydrate synthesis. This occurs via gluconeogenesis during prolonged fasting (starvation) when triglyceride

turnover and release of glycerol from fat metabolism are low (Moon and Johnston, 1980). Since body fat levels in small mammals can be consistently low throughout the year, *M. rutilus* may utilize protein to meet energy requirements by catabolizing muscle or digestive tissues (which in turn would lower its body mass and the absolute energy requirements necessary to maintain these excess tissues). One prior study showed decreases in water percentage of lean mass and organs, with these two components explaining nearly all the seasonal variation in total body mass (Zuercher *et al.*, 1999).

An increased cell size or a greater number of cells in an organ might provide cumulative advantages in some seasons for *M. rutilus*. Mass allocation translates roughly into energy allocation, as there is a substantial energy cost per gram of organ tissue. Thus, a seasonal change in the size or mass allocated to a given organ infers a change in the importance of that organ in one season over another. For example, having a longer digestive tract with more microvilli in summer would maximize digestion, energy absorption, and body mass when food availability and activity are both at their peak (much like pythons, "sit and wait" predators, which degrade their digestive tracts, but rebuild them post-prey capture (Secor and Diamond, 2005)). However, lower quality foods in winter may require longer digestive tracts to increase assimilation efficiency. Reduced movement and low intake of fatty acids in winter might also, for example, reduce the need for maintaining glycogen stores, liver enzymes, and bile salts (Moon and Johnston, 1980). Decreasing the size or number of cells producing those substances may lead to a seasonal reduction in liver size, while kidney size might increase in winter in response to either an increase in evaporative water loss or a decrease in free water intake. Environmental, behavioral, dietary, and social factors are all likely to act as selective pressures on small mammals in highly seasonal environments to regulate ideal body composition and physiological processes.

The daily energy expenditure or field metabolic (FMR) of animals can be

estimated through use of the doubly labeled water (DLW) method (Lifson and McClintock, 1966; Nagy, 1983; Speakman, 1997). The loss of CO_2 from a free-living animal over an extended period is directly proportional to its rate of energy expenditure during that time and can therefore be used as an indirect measurement FMR. The DLW method has been well established (Nagy, 1989; Speakman, 1997), and has been used in many species to determine whether energy expenditure differs in seasons (*e.g.*, Holleman *et al.*, 1982). Upon entering the body, hydrogen and oxygen inputs undergo a complete mixture with the body water pool through reactions with carbonic anhydrase. By injecting water that contains a known volume (or mass) of labeled isotopes of oxygen (^{18}O) and hydrogen (^{2}H), CO_2 production can be measured in a free-living animal. As the rate of ^{18}O loss roughly equals the combined rate of water and CO_2 loss, and the rate of ^{2}H loss roughly equals the rate of water loss, the difference between these two rates can be used to calculate CO_2 loss.

5.3 Methods

Voles were live and lethal trapped in grids of mixed spruce and birch forest in Chugach State Park, Alaska (61'17"43°N, 149'32'10°W) from 2004-2007 using Sherman and snap traps. The live-trapped animals were used for the field metabolic rate (FMR) analysis, while lethal trapped animals were used in the analyses of body composition and organ mass. Animals were captured and treated following procedures approved by the American Society of Mammalogists (ASM Animal Care and Use Committee, 1998), and all procedures were approved by the University of Alaska Anchorage Institutional Animal Care and Use Committee (IACUC) committee (protocol# 2005VanTe1), the Chugach State Park Service, and the Alaska Department of Fish and Game.

Adult *M. rutilus* were collected from Chugach State Park, Alaska between November 2004 and January 2007 and were grouped into seasons (Zuercher *et al.*, 1999; Stevenson *et al.*, 2009a). Seasons were designated as spring (April-May),

early summer (June-July), late summer (August-September), fall (October-November), and winter (early and late winter combined, December-March). A mass threshold of 17.5g has historically been used to distinguish adults from younger individuals in early and late summer for this species with all other individuals outside of these seasons considered to be of adult age (Whitney, 1976; Zuercher *et al.*, 1999; Stevenson *et al.*, 2009b). During the study period, breeding in Chugach State Park began in mid-April and ceased between late August and early September, with peak reproduction occurring in spring and early summer seasons (Stevenson *et al.*, 2009b).

Photoperiod (hours of daylight) was recorded on each capture date. Average ambient temperature (T_a) was recorded hourly by data loggers (Hobo, Inc.) that were placed at a height of 2m within a few meters of my trapping sites (there was a malfunction in sensors initially placed at ground level under the snow, which caused us to rely upon above-ground sensors). I used data from National Resources Conservation Services (NRCS) snowpack telemetry (SNOTEL) sites in Chugach State Park, Alaska (Anchorage Hillside) and Moraine, Alaska to confirm ambient temperature measurements and obtain levels of snow depth and precipitation.

5.3.1 Body Composition and Organ Masses

The body condition of lethal-trapped voles was assessed using a dual-energy X-ray absorptiometry (DXA) apparatus (Lunar/GE PIXI Mus2) that had been previously validated for use in small rodents, including *M. rutilus* (Nagy and Clair, 2000; Johnston *et al.*, 2005; Stevenson and van Tets, 2008). I measured and compared body composition percentages (fat, LM, BW, protein, and mineral) in up to 114 voles at different times of the year to test for seasonal differences in these percentages and to identify the environmental factors most strongly linked to any seasonal changes. Vole carcasses were thawed, placed on the DXA platform, and scanned. DXA values were then transformed into accurate values

using corrective equations for percentages of fat, LM, BW, protein, and mineral (Stevenson and van Tets, 2008). Only body mass required log transformation.

I dissected the heart, liver, spleen, stomach, small intestine, large intestine, cecum, and kidneys from each scanned carcass. Organs were then dried overnight at 60°C. Relative dry mass was determined for each organ by dividing its dry mass by total body mass. The contents of the digestive organs were removed before weighing by flushing with 0.9% NaCl solution. Lengths of intestines were measured by stretching them to a maximum length without breaking, marking the endpoints, and measuring the distance. Cecum and kidney data were normalized prior to statistical analyses by arcsine transformations. I measured and compared the relative dry masses of these visceral organs in up to 109 voles at different times of the year to test for seasonal differences in the masses of these organs and to identify the environmental factors more strongly linked to any seasonal changes.

5.3.2 Field Metabolic Rate

Voles were live-trapped in Chugach State Park, Alaska between 2004 and 2007. I measured the field metabolic rate (FMR, ml CO_2 g^{-1} h^{-1}) of voles in breeding (n = 7) and non-breeding (n = 4) seasons using the doubly labeled water (DLW) technique (Lifson and McClintock, 1966; Butler *et al.*, 2004). This method has been previously validated by comparison to indirect calorimetry in a range of small mammals (*e.g.,* Speakman and Krol, 2005). Animals were weighed (±0.1 g portable balance), and a 100µL blood sample was obtained from tail clip of non-study animals to estimate the background isotope enrichments of ^{2}H and ^{18}O (method C, Speakman and Racey, 1987). Water was distilled from blood overnight in heat-sealed Pasteur pipettes, immediately re-sealed into small glass tubes formed from the sealed pipette tips with minimal head-space, and stored at room temperature (20°C). Study animals were injected with a known mass of DLW (approximately 66% H_2O; 22% $H_2{}^{18}O$, 95 % enriched; 11.1%

$^{2}H_2O$, 99.5% enriched; administered IP, approximately 1g/100g body weight). Syringes were weighed in the lab before and after administration using a balance (±0.0001g) to calculate the mass of DLW injected. Blood samples were taken after one hour of isotope equilibration to estimate initial isotope enrichments (Krol and Speakman, 1999). Animals were recaptured 1-2 days post dose, and final blood samples (100µl) were taken as close as feasible to whole 24 h periods (Speakman and Racey, 1988) to estimate isotope elimination rates. The isotope ratios ^{18}O: ^{16}O and ^{2}H: ^{1}H were analyzed using gas source isotope ratio mass spectrometry (Finnigan Delta Plus XD, TC/EA) at the University of Alaska Anchorage ENRI Stable Isotope facility. Isotope enrichments were converted to values of FMR following Coward *et al.* (1985) and Speakman (1997). I used this technique to test whether the FMR of free-living voles was different in the breeding and the non-breeding season, and whether it was significantly correlated with ambient temperature.

5.3.3 Statistical Analyses

SPSS (v16.0) statistical software was used for all statistical analyses. Analyses of variance (ANOVA) with Tukey's HSD post-hoc tests were used to compare relative values for body composition and visceral organ mass in different seasons. I used multiple regression analyses with stepwise variable entry to determine which proximate environmental factors (photoperiod, ambient temperature, snow depth, or daily precipitation) contributed to the highest amounts of unexplained variance in body composition, organ mass, and FMR. I tested for differences in FMR of voles between a generalized breeding season (April 1st to August 25th) and a non-breeding season (August 26th to March 31st) (Stevenson *et al.*, 2009b). I also used this technique to test whether FMR was correlated with ambient temperature.

5.4 Results

Several components of body composition changed seasonally (ANOVA, $p < 0.05$, adult animals, sexes grouped together). Body mass was highest in early summer (25.1 ± 1.0g), but lowest in winter (13.5 ± 1.0g) ($p < 0.001$) (Fig. 5.1a). Percentage fat, however, was the only element of condition that did not change seasonally ($F_{[3,114]} = 1.569$; $p = 0.187$, Fig. 5.1b). Percentage LM and %BW were highest in late summer, but lowest in spring and fall (Fig. 5.1c and 5.1d, respectively). Percentage protein was highest in early summer and late summer and lowest in winter (Fig. 5.1e). Percentage mineral was highest in fall and winter and lowest in early summer and late summer (Fig. 5.1f).

Multiple regression analyses determined that the factors most strongly related to body composition were photoperiod, snow depth, and gender (Table 5.1). Photoperiod, however, was the only factor that accounted for more than 25% of the unexplained variance in highly significant ($p < 0.001$) models (for the entire model, Body mass $R^2 = 0.65$; %Protein $R^2 = 0.33$; %Mineral $R^2 = 0.43$).

There were significant ($p < 0.05$) seasonal changes in the relative dry mass (g dry organ/ g body mass) of all visceral organs except kidneys, small intestines, and large intestines (although both intestines showed significant seasonal change in their relative lengths) (Fig. 5.2). The relative dry mass of the heart was highest in winter and lowest in early summer and late summer. Values for liver were highest in late summer and lowest in spring, fall, and winter. Spleen values were highest in late summer and lowest in spring and winter. Stomach values were highest in spring and lowest in early summer, late summer, and fall (Fig. 5.2d). Relative dry small and large intestine masses did not change seasonally ($p = 0.176$ and $p = 0.055$, respectively, Fig. 5.2e). Similar to changes in stomach mass, the relative cecum masses were highest in spring and lowest in early summer, late summer, and fall. Relative dry paired kidney mass did not change seasonally ($p = 0.08$, Fig. 5.2g). Despite no significant change in relative dry mass of either intestine, the relative lengths of the intestines (cm intestine length/ cm body

length) did change seasonally ($p < 0.05$). Small intestines were longest in late summer and shortest in spring, early summer, and winter. Large intestines were longest in late summer, fall, and winter and shortest in spring and early summer.

Multiple regression analyses determined that the environmental factors most strongly related to relative organ masses were T_a, gender, snow depth, and precipitation (Table 5.2). However, T_a and gender were the only factors that accounted for > 25% of the unexplained variance in highly significant ($p < 0.001$) models (for entire model, Heart $R^2 = 0.30$; Small intestine mass $R^2 = 0.48$; Large intestine mass $R^2 = 0.36$). Relative heart mass was most strongly predicted by T_a, whereas both intestinal masses were most strongly predicted by gender. Alimentary canal length was higher in females overall (Small intestine, n = 63, Females 6.27 ± .11, Males 5.88 ± .12; Large intestine, n = 67, Females 2.67 ± .04, Males 2.57 ± .06).

Mean values for FMR in voles (Breeding: n = 7, 3.1237 ± 0.6032; Non-breeding: n = 4, 3.2870 ± .3260) did not differ significantly with respect to breeding season or sex (ANCOVA, fixed factors = breeding season and sex, covariate = T_a, $F_{[3,10]} = 2.465$, Corrected Model $p = 0.155$). The FMRs of breeders and non-breeders did not differ, although the variance of voles in the breeding season was almost double that of non-breeding voles. Field metabolic rate was significantly correlated with T_a (linear regression $y = -0.1165x + 3.7913$, $R^2 = 0.29$, $p = 0.037$) when sex and breeding season were combined (Fig. 5.3).

5.5 Discussion

The strong seasonality of high-latitude regions favors seasonal changes in the allocation of resources to different tissues over others. The strong seasonal change in body mass is likely to have caused some relative organ mass values to show a direct inverse pattern, namely mineral content (Fig. 5.1f), heart, stomach, and cecum, (Figs. 5.2a, 5.2d, and 5.2g, respectively). Some elements of body composition and certain organ masses did not show seasonal change, namely

percentage fat, and relative dry mass of kidneys and intestines. However, absolute fat mass was directly correlated with photoperiod and body mass, and it was highest during long days and lowest during short days (see Stevenson *et al.*, 2009b for links between mass, fat content, and photoperiod).

Notable winter decreases in body composition and relative visceral organ mass included percentage protein and the relative masses of the liver and the spleen, while winter increases included mineral content, the heart, and the stomach. The severe winter decline in protein is in agreement with other studies in which northern red-backed voles appeared to fuel part of winter activity through gluconeogenesis and use of protein stores (Zuercher *et al.*, 1999). The winter decline in the liver and spleen masses were also not surprising. If the glycogen and free-fatty acid stores are low, and the voles are relying more on protein stores in winter, diversion of maintenance metabolism from the liver to thermoregulation or maintenance metabolism would be beneficial. Gluconeogenesis does occur in the liver, but liver is also used for glycogen storage, detoxification, and production of biochemicals necessary for digestion – all of which could be of less importance in winter. Similarly, immune function is costly, and the voles may be taking an "all or nothing" approach, investing large amounts of energy into maintaining large spleens in summer, but diverting energy away from maintaining these cells in winter (discussed in detail further, below). The percentage of protein, and the relative masses of the liver and spleen all increased in summer as home range and energy availability increased. In winter, the mineral content and the relative dry mass of the heart and stomach increased. These values, along with cecum weight, decreased in summer.

Small intestine length increased in summer, suggesting a greater dependence upon the length of the alimentary tract during this season than in other seasons. Female alimentary tract length superceded that of males across all seasons, presumably reflecting the more expensive cost of reproduction to female voles and the need to acquire more energy in the breeding season. Seasonal changes in

relative lengths, but not masses, of intestines, suggest that lengthening of the gut is more advantageous than simply adding intestinal mass. Voles might, therefore, get a return on their seasonal investment into the alimentary tract by extracting more energy from food that resides in the gut for longer periods of time (although the actual energy intake depends on several other factors, including digestibility of the diet, length of time, and gut capacity). Interestingly, stomach and cecum values were much higher in spring than any other season. This may be related to the need for digestion of different food types and a greater dependence upon fermentation. A large cecum in spring is presumably quite important in helping voles to extract energy in this season (probably from different food sources, depending on the climate and snow cover) while intestinal length is not yet maximal.

Relative kidney weights did not undergo significant seasonal change ($p = 0.08$, Fig. 5.2g). Increased sample size or more rigidly defined sampling time within season might indentify seasonal differences, as kidneys change seasonally in other species of voles (*e.g.*, *M. rufocanus*, Bolshakov, 1989). Values were slightly higher in late summer and slightly lower in fall and winter. Percentage body water also did not decrease, reflecting the fact that tissue hydration state is strongly defended, even though both heat and water vapor flux from the mouth increase during exhalation at low temperatures. The cost of ingesting frozen water and heating it appears, therefore, to be less deleterious to *M. rutilus* than the cost of BW fluctuation and/or the cost of altering renal structures to deal with dehydration.

The strong seasonality of high-latitude regions usually requires the allocation of energy to either reproduction or thermoregulation. Winter breeding or presence of newborn pups in winter have, however, been observed on rare occasions in almost every species studied (*e.g.*, Hansson, 1984; Kaikusalo and Tast, 1984; Nelson, 1987; Bronson and Heideman, 1994; Gockel and Ruf, 2001; Millar, 2001). The results from this study support the assumption that an energetic

bottleneck exists and will usually keep breeding and overwintering seasons separate. Values for FMR of voles in breeding and non-breeding seasons were equal, although the variance of voles in the breeding season was much higher. In this study, the highest value recorded for energy expenditure was for a reproductive male at cold temperature (captured in mid-April while snow was on the ground, melting during the day and freezing at night). The fact that this animal had recrudescing testes, was producing germ cells, and was maintaining a constant body temperature in average subzero temperatures in the presence of free water from snowmelt probably contributed to its high FMR (Fig. 5.3).

The FMR of voles in my study ranged from 1.5 to 5.8 ml CO2 $g^{-1}h^{-1}$. A prior study on free-living *M. rutilus* in interior Alaska (where average ambient temperature is much higher and winter ambient temperature and snow pack are much lower than in southcentral Alaska) reported FMRs for this species as highest in summer (6.7 ml CO2 $g^{-1}h^{-1}$) and winter (7.6 ml CO2 $g^{-1}h^{-1}$) and lowest in spring (5.1 ml CO2 $g^{-1}h^{-1}$) and fall (6.4 ml CO2 $g^{-1}h^{-1}$) (Holleman *et al.*, 1982). In this same study, it was determined that free-living voles had FMRs that were approximately 1.73, 1.47, and 1.40 times that of laboratory-raised voles' basal metabolic rates in the same spring, summer, and fall seasons, respectively (both metabolic rates were calculated using the DLW method, and both were calculated as ml/g FFBW·h). Technology and methods used in the DLW method have changed substantially since this study almost 30 years ago, but assuming that these metabolic ratios hold for my study, as well (*i.e.*, FMR is ~ 1.5 times that of BMR in each season), support for an energetic bottleneck might actually be somewhat lacking with regard to what has traditionally been believed (although no comparisons of winter values were made between FMR and BMR in the prior study). In my study, the FMR of males did not differ significantly from females, which is also in agreement with Holleman *et al.* (1982).

The influence of snow depth in several multiple regression models may be related to its influence as a thermal barrier and relevance to the subnivean

microclimate in which the voles spend their winters. Snow depth may thus be thought of as an indirect correlate of subnivean ground temperature, and, arguably, a more accurate one than ambient temperature, which was recorded at a height of 2m. Snow depth and subnivean ground temperature are inversely related, and arvicoline rodents are generally thought to spend most of their time in the subnivean space in winter to gain the maximum thermal benefit of this insulation (hence, snowdepth's slight influence on several models related to body composition (Table 5.1) and relative visceral organ mass (Table 5.2)). The unpredictable winter freeze-thaw events characteristic of southcentral Alaska may also increase the importance of snow depth as a factor relative to ambient temperature. Yet despite it being a frequent minor factor, snow depth was only slightly related to changes in allocation of body composition and relative organ mass and was never a dominant factor for models contributing to greater than 25% of the unexplained variance. It may be that voles are able to escape harmful effects of snow melt and minimize energy use.

Melted snow also likely has a negative effect on reproductive condition, as arvicolines in tundra areas have been shown to restrict breeding during first snowfall and snowmelt (Millar, 2001). Voles in my study populations did not wait for complete snow melt in spring to begin breeding (Stevenson *et al.*, 2009b), which may be related to the milder climate of southcentral Alaska or to various niches available for escaping melting snow relative to tundra.landscape (*e.g.*, nest locations under fallen brush, in fallen logs, around root system and trunks of trees, within protected moss beds or temporary escapement to the branch of a tree or bush). It is also possible that much of the unexplained variance in composition and organ size is attributed to diet, as it can be quite diverse in food substances (Bangs, 1984), and is likely to change seasonally (A. Brennan, pers. comm.)

Finally, the immune system of mammals may be compromised during high periods of stress or maximal reproductive effort. In the laboratory, short days alone are known to upregulate immune function, but the interaction of short days

and temperatures downregulate it (Nelson *et al.*, 1998). The suppressive effect of testosterone on immune function is also hypothesized to be one reason why males have lower immune responses than females (Klein and Nelson, 1998). Corticosterone (CORT) is known to increase in response to noxious stimuli, and is already highest during the breeding and post-breeding seasons in mammals (Romero, 2002). Chronically elevated testosterone and CORT towards the end of an exhaustive breeding summer might also compromise immune function (Klein and Nelson, 1998). Proliferation of splenocytes (*i.e.*, any of the different white blood cell types) or increased splenic mass may be highest during times when voles are most susceptible to disease and able to invest in an immune response. The occurrence of hypertrophic spleens within populations has been regarded as an indicator of the presence of damaging factors in three species of *Myodes* (formerly *Clethrionomys*), but not necessarily in *Microtus* or certain mouse species (Olenev and Pasichnik, 2003). Spleens in my study populations were noticeably larger (possibly hypertrophic) in late summer. This is could be due to overactivity of its macrophage system, but could also be due to higher blood volume at this time of year, especially in pregnant or lactating females.

5.6 Conclusions

Voles underwent seasonal changes in body composition, relative organ mass, and field metabolic rate that were related to environmental parameters. Most notably, values for percent protein, liver, and spleen increased in winter, while percent mineral, heart, and stomach decreased. In summer, percent protein, liver, spleen, and large intestine length increased. Cecum and stomach values were highest in spring, reflective of increasing energy requirements in spring. Body condition was most strongly related to photoperiod, while organ masses varied by gender and ambient temperature. Snow depth, which is reflective of voles' winter microclimate, had a significant, but minor positive effect on most parameters. Field metabolic rate did not differ between breeding and non-breeding seasons,

but was significantly correlated with T_a. Taken together, these results suggest that changes in climate will have a substantial effect on vole physiology and energy allocation.

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5.8 Tables

Table 5.1 Photoperiod Contributes to Greater Than 25% of the Unexplained Variance in Body Mass, Percentage Protein, and Percentage Mineral. Multiple regression tests were used to determine whether environmental factors were linked to different body composition parameters. Factors accounting for more than 25% of the unexplained variance in body composition are in bold. When multiple factors contributed to a significant model, the dominant factor is marked with an asterisk (*).

Multiple Regression Models - Body Condition

Independ. Vars. Entered: Snow Depth, Amb. Temp., Precipitation, Photoper., Gender

Dependent Variable	df	R^2	F	p-value of Model	Const. B_c	Factors Included in Model
Body Mass	130	**0.650**	118.73	<0.001	1.072	**Photoperiod*** ($p < 0.001$) Snow Depth ($p < 0.001$)
%Fat	118	0.128	8.52	<0.001	4.760	Gender* ($p = 0.004$) Snow Depth ($p = 0.008$)
%LM	119	0.054	6.70	0.011	90.249	Snow Depth ($p = 0.011$)
%Body Water	119	0.053	6.58	0.012	70.762	Snow Depth ($p = 0.012$)
%Protein	119	**0.333**	29.14	<0.001	16.861	**Photoperiod*** ($p < 0.001$) Snow Depth ($p = 0.001$)
%Mineral	114	**0.434**	42.94	<0.001	4.986	**Photoperiod*** ($p < 0.001$), Snow Depth ($p = 0.004$)

Table 5.2 Ambient Temperature and Gender Contribute to Greater Than 25% of the Unexplained Variance in Heart and Intestinal Mass, Respectively. Multiple regression tests were used to determine whether environmental factors were linked to different relative organ masses. Factors accounting for more than 25% of the unexplained variance in organ mass are in bold. Where multiple factors contributed to a significant model, the dominant factor is marked with an asterisk (*).

Multiple Regression Models - Relative Dry Visceral Organ Mass

Independ. Vars. Entered: Snow Depth, Amb. Temp., Precip., Photoper., Gender

Dependent Variable	df	R^2	F	p-value of Model	Const. B_c	Sig. Factors Incl. in Model
Heart	118	**0.304**	38.502	<0.001	0.003	**Amb. Temp.*** ($p < 0.001$), Gender ($p = 0.003$)
Liver	116	0.131	17.372	<0.002	0.015	Snow Depth ($p < 0.001$)
Spleen	115	0.056	6.728	0.011	0.048	Snow Depth ($p = 0.011$)
Stomach	112	0.178	11.942	<0.001	0.002	Snow Depth ($p < 0.001$) Gender ($p = 0.035$)
SI	113	**0.475**	16.151	<0.001	0.004	**Gender*** ($p < 0.001$) Snow Depth ($p = 0.003$)
LI	115	**0.361**	8.49	<0.001	0.001	**Gender*** ($p < 0.001$) Amb.Temp. ($p = 0.003$)
Cecum	115	0.116	14.891	<0.001	0.050	Snow Depth ($p < 0.001$)
SI Length	109	0.155	9.89	<0.001	6.13	Snow Depth* ($p < 0.001$) Precipitaiton ($p = 0.040$)
LI Length	114	0.155	20.732	<0.001	3.067	Photoperiod ($p < 0.001$)

Non-significant models: Kidneys

5.9 Figures

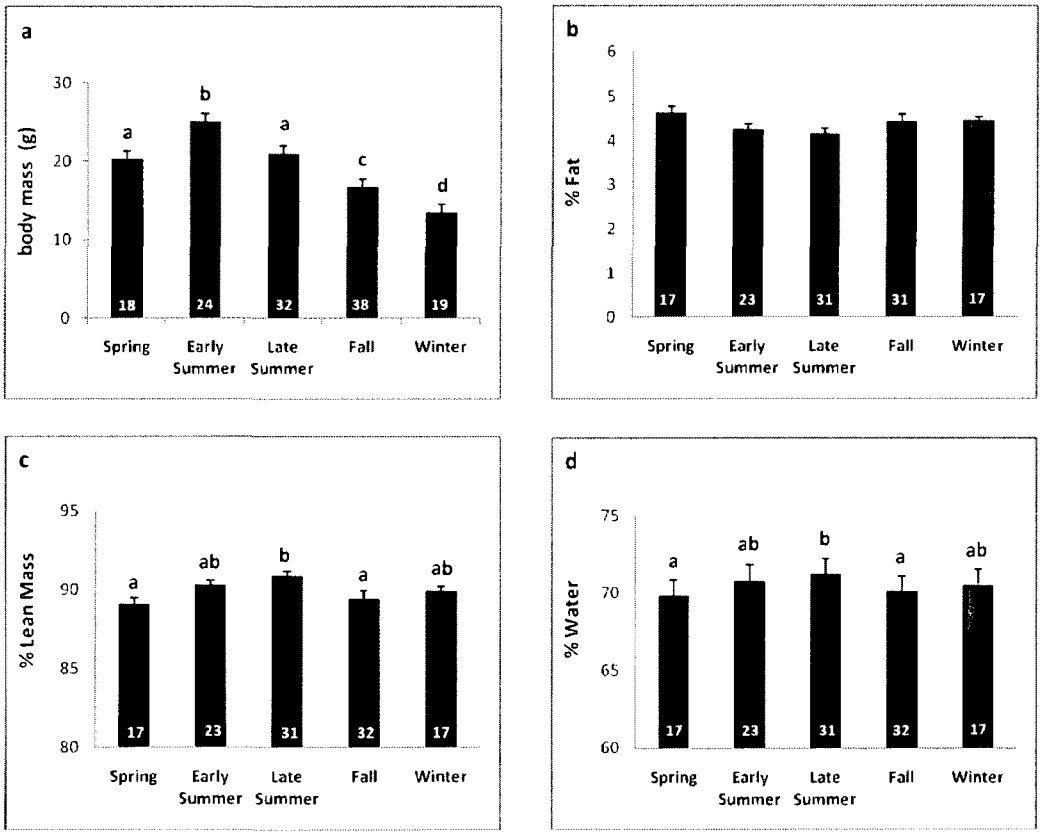


Figure 5.1 The Seasonality of Body Composition in Northern Red-backed Voles (*Microtus rutilus*) in Southcentral Alaska. Bars represent mean values and error bars represent SEs for (*a*) body mass (wet), (*b*) percentage body fat, (*c*) percentage lean mass, (*d*) percentage body water, (*e*) percentage protein, and (*f*) percentage mineral. The sample size (n) for each season is displayed within the corresponding bar above the x-axis. Mean values represented by bars that share the same letter are not significantly different ($p > 0.05$). Except for percentage fat, all components of body condition showed significant seasonal change (ANOVA, $p < 0.05$).

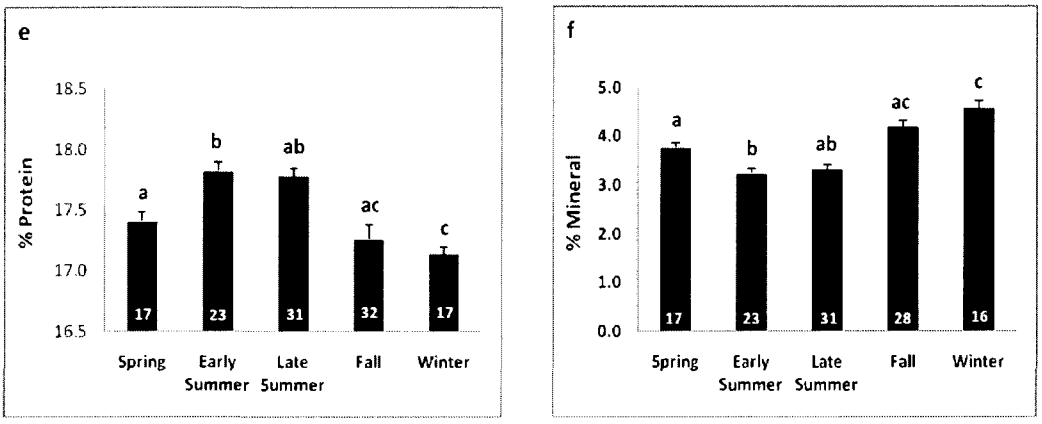


Figure 5.1 Continued...

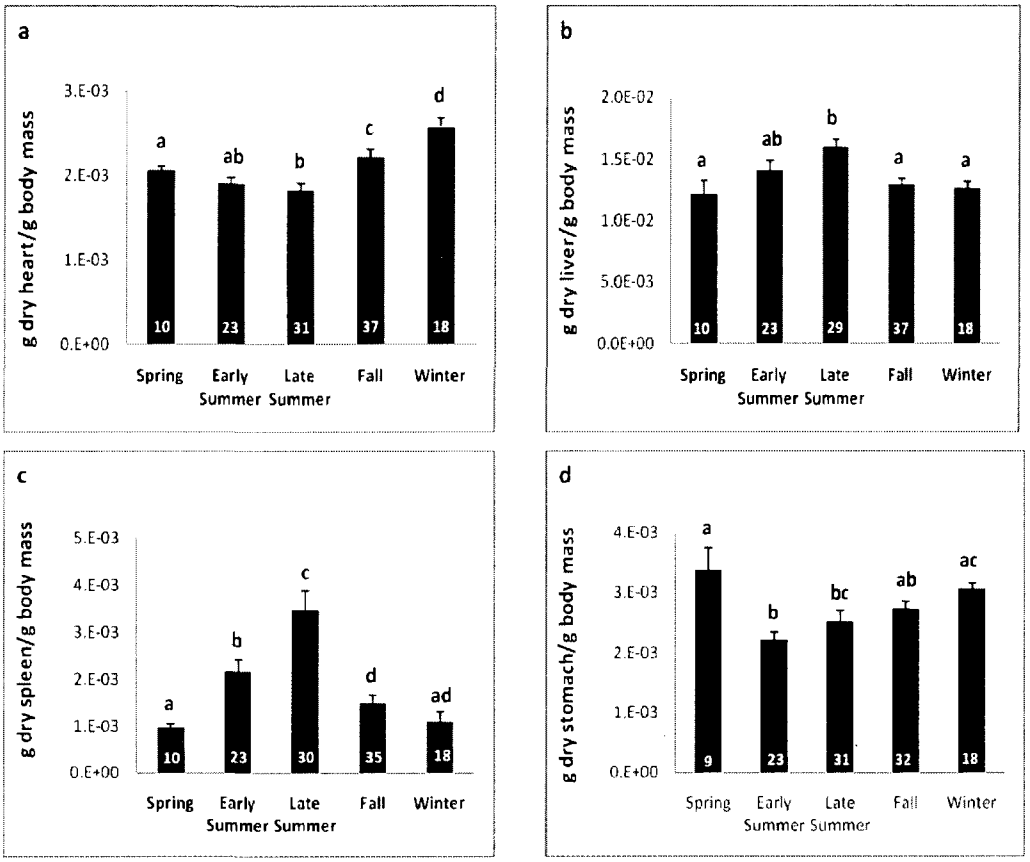
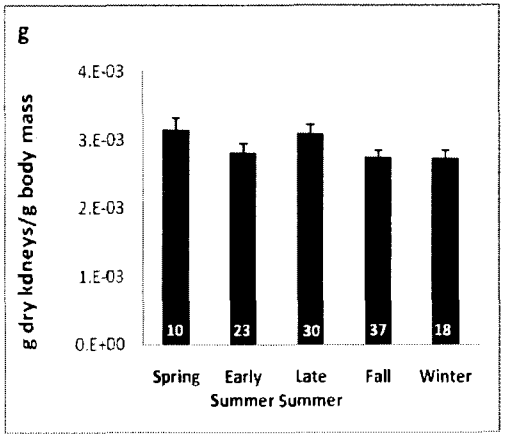


Figure 5.2 The Seasonality of Relative Visceral Organ Size in Northern Red-backed Voles (*Microtus rutilus*) in Southcentral Alaska. Bars represent mean values and error bars represent SEs. for relative dry masses (g dried organ/ g of body mass) for (*a*) heart, (*b*) liver, (*c*) spleen, (*d*) stomach, (*e*) small and large intestine (SI and LI, respectively), (*f*) cecum, and (*f*) paired kidney. Mean values and SEs are also shown for (*h*) relative lengths of intestines. The sample size (n) for each season is displayed within the corresponding bar above the x-axis. Mean values represented by bars that share the same letter in the same color are not significantly different ($p > 0.05$). Except for relative dry masses of SI, LI, and kidneys, all visceral organs showed significant seasonal change (ANOVA, $p < 0.05$). Relative SI and LI also changed seasonally, despite no significant seasonal change in organ mass.



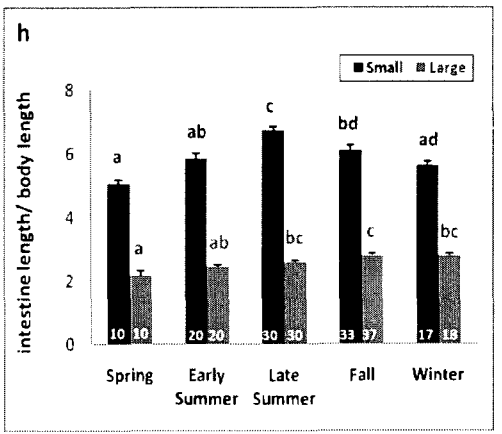
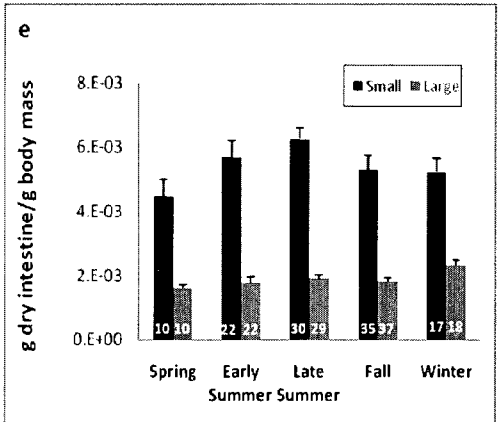


Figure 5.2 Continued…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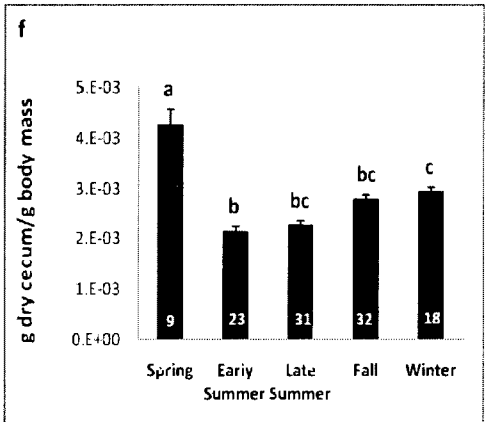
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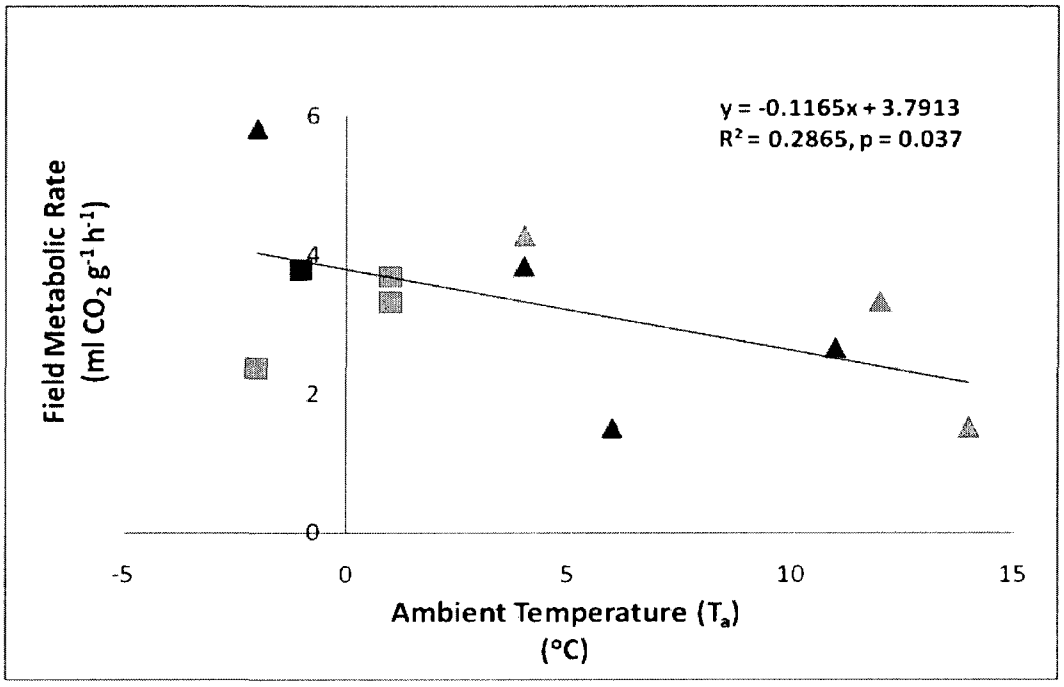

Figure 5.3 The Field Metabolic Rate (FMR) of Northern Red-backed Vole (*Myodes rutilus*) in Southcentral Alaska Is Significantly Correlated With Ambient Temperature (T_a). Breeding males (black triangles), non-breeding males (black squares), breeding females (gray triangles), and non-breeding females (gray squares) are shown. There was a significant relationship between FMR and T_a, regardless of gender or reproductive state.

Chapter 6:

Bone Mineral Density Changes Seasonally in a Nonhibernating Alaskan Rodent, the Northern Red-backed Vole (*Myodes rutilus*)[1]

6.1 Abstract

High-latitude voles and lemmings undergo strong seasonal changes in their behavior and physiology, which may lead to concurrent changes in bone mineral density (BMD). I tested whether the BMD of northern red-backed voles (*Myodes rutilus*) in Alaska changed seasonally, and if so, whether these changes in their weight-bearing bones were correlated with seasonal changes in photoperiod (a mediator of activity and concentrations of reproductive hormones in high-latitude voles and lemmings), body mass, body length, or a combination of these. I used dual-energy X-ray absorptiometry to measure the BMD of the femur and humerus of voles collected in different seasons. BMDs increased dramatically from the start of spring to their peak level in early summer, and then decreased gradually to their lowest point in late winter. BMDs were significantly lower in fall and winter than in spring and early summer. BMDs of long bones were significantly correlated with both body mass and photoperiod, which accounted for 46.2% and 45.7% of the variation in the BMDs of femur and humerus, respectively. The strong changes that I observed in BMD are likely to be due, in part, to the combined effects of strong seasonal changes in body mass, activity, and baseline levels of reproductive hormones.

6.2. Introduction

Arvicoline rodents (voles and lemmings) in high-latitude regions exhibit strong seasonal changes in their behavior and physiology that could affect bone mineral density (BMD). Breeding begins in spring under increasing photoperiod

[1] Stevenson KT, van Tets IG, Chon DY. 2009. Making no bones about it: Bone mineral density changes seasonally in a non-hibernating Alaskan rodent. *J Mammal* 90(1):25-31.

in most species and is marked by notable increases in activity (Stebbins, 1974), home-range territory (West, 1977), body mass (Nay *et al.*, 2007; Sealander, 1967; Zuercher *et al.*, 1999), and concentrations of sex steroids (Wallen and Schneider, 2000). Winter is the reverse, and breeding typically ends under decreasing photoperiod. The spring–summer breeding season of most high-latitude voles and lemmings is generally fast-paced and includes the production of multiple litters. In fact, some animals born in summer may even reach adulthood and breed within the same year of their birth (Prendergast *et al.*, 2001; Whitaker, 1996). In winter, nonhibernating arctic and subarctic mammals remain active, but exhibit physiological acclimatization (Nay *et al.*, 2007; Sealander, 1967; 1972; Zuercher *et al.*, 1999), changes in social behavior (West, 1977; Wolff and Lidicker, 1981), and increased energy expenditure (Holleman *et al.*, 1982) to offset thermoregulatory costs.

The winter reductions in body mass, movement, and gonadal sex steroids described above could contribute to either a seasonal osteopenia (a condition of reduced bone density below normal peak BMD) or seasonal osteoporosis (a more severe weakening of bone due to excessive loss of protein and mineral content) in the weight-bearing long bones. Bone is a dynamic tissue that is constantly reshaped by osteoblasts, which build bone, and osteoclasts, which resorb bone. In mammals, reductions in bone density can result from restricted movement and a lack of mechanical stress on bones (disuse osteoporosis — Blouin *et al.*, 2007; Resnick, 1988), prolonged spaceflight (a special case of disuse osteoporosis— LeBlanc *et al.*, 2000; Milstead *et al.*, 2004), changes in diet or mineral uptake (Demigne *et al.*, 2006; Gennari, 2001; Rodriguez-Martinez and Garcia-Cohen, 2002), reductions in baseline concentrations of gonadal steroids and growth hormone (Christmas *et al.*, 2002), or changes in life-history stage (Bonnick, 2006; Genarri, 2001).

Although arvicoline rodents remain active year-round in the subnivean space (Getz *et al.*, 2005; Holleman *et al.*, 1982; Stebbins, 1974), their activity and home

range are severely reduced in winter. Even at lower latitudes, both meadow voles (*Microtus pennsylvanicus*) and prairie voles (*M. ochrogaster*) restrict movement in winter, sometimes covering a distance 2–3 times less than that covered in other seasons (Getz *et al.*, 2005). The free-living arctic–subarctic northern red-backed vole (*Myodes* [formerly *Clethrionomys*] *rutilus*) has been described as having an activity pattern that is moderately high in spring, extremely high in summer, and extremely low in winter (Stebbins, 1974); and Tavernier *et al.* (2004) have shown that the activity patterns of this species in a laboratory setting are strongly linked with photoperiod. These photoperiod-induced reductions in activity could drive changes in bone density.

In Alaska, both *M. rutilus* and the taiga vole (*Microtus xanthognathus*) display pronounced midwinter aggregation (communal nesting) that is associated with severely restricted movement and home range (West, 1977; Wolff and Lidicker, 1981). Communal nesting may occur as a result of reproductive cessation, reduced aggression, clumped food resources, a scarcity of overwintering sites, thin snowpack, low temperatures, or other factors (West and Dublin, 1984). It also can provide advantages, such as food sharing, thermoregulatory benefits from huddling, and cooperative defense (Wolff and Lidicker, 1981). When voles huddle together in one large ''vole complex'' in a nest, it allows them to lower their energy maintenance requirements. While huddled as a unit, they gain the corporate benefits of a lower surface area-to-mass ratio and shared thermoregulatory costs, but they also retain the individual benefit of a lower overall maintenance metabolism by decreasing body mass and internal organ masses during what is likely a nutrient-poor season (Nay *et al.*, 2007;Sealander, 1967; Zuercher *et al.*, 1999). In winter, movement of individuals is likely to be inhibited or restricted by snow and ice accumulation, the possibility of increased conductive and convective heat loss, the energetic cost of tunneling through the subnivean space, and a higher risk of predation above the snow. If food quality and mineral content are consistently poor but consistently available throughout

the environment, it would seem advantageous for small, nonhibernating mammals to restrict movement, as well as tissue, organ, and body mass as much as possible in winter.

Additionally, a photoperiod has a strong effect upon the endocrine function of voles and lemmings. The hypothalamus–pituitary–gonad axis is affected by melatonin release, which is triggered by changes in day length (Goldman and Nelson, 1993; Kriegsfeld and Nelson, 1996; Meek and Lee, 1994; Wallen and Schneider, 2000). Although little research has been conducted on bone densities of free-living rodents, a large body of clinical evidence suggests that these changes likely have some influence on BMD. In male human subjects, for instance, the interaction of growth hormone and testosterone promotes bone growth, whereas estrogen replacement and growth hormone supplementation have been shown to slow bone loss in menopausal women (Christmas *et al.*, 2002). However, growth hormone does not appear to be affected by photoperiod in other tested mammalian species (Bartke *et al.*, 1980; Borer *et al.*, 1982; Kendall *et al.*, 2003; Klemcke *et al.*, 1983). The aim of this study was to test whether the BMD of the northern red-backed vole changed seasonally, and if so, whether seasonal changes in BMD in its weight-bearing bones were correlated with seasonal changes in body morphology and photoperiod.

6.3 Methods

Adults of *M. rutilus* were collected from Chugach State Park, Alaska between November 2004 and January 2007 and were grouped into six seasons similar to those of Zuercher *et al.* (1999). Seasons were designated as spring (April–May), early summer (June–July), late summer (August–September), fall (October–November), early winter (December–January), and late winter (February–March). For comparisons between genders, I followed Zuercher *et al.* (1999) by collapsing early winter and late winter into one category (''winter'') and early summer and

late summer into another category (''summer'') to eliminate the seasons with the smallest adult sample size (late summer and late winter).

During the study period, breeding in Chugach State Park began in mid-April and ceased between late August and early September. Peak reproduction seasons were in spring and early summer (Nay *et al.*, 2007). Like other arvicoline rodents, northern red-backed voles mature rapidly, weaning after approximately 19 days (Tavernier *et al.*, 2004) and reaching adulthood at around 45 days (Prendergast *et al.*, 2001; K. T. Stevenson, pers. obs. on *M. rutilus*). A mass threshold of 17.5 g has historically been used to distinguish adults from younger individuals in early and late summer. Outside of these seasons, all animals have been considered to be of adult age (Whitney, 1976; Zuercher *et al.*, 1999). To exclude any possibility that animals of adult age retaining juvenile characteristics might be included in my analyses, I excluded animals weighing < 20 g in these seasons. I did not observe any animals in breeding condition during winter, nor did I see any evidence of young born in winter.

Carcasses were weighed at the time of capture and were frozen at -20°C. I later dissected each vole, determined gender by the presence of either testes or ovaries, and removed the left humerus and femur. The length of each bone was determined using digital calipers accurate to the nearest 0.01 mm. Bone densities were determined using a PIXImus2 dual-energy X-ray apparatus (Lunar/GE, Madison, Wisconsin) that was known to accurately predict bone measurements in rodents (Nagy and Clair, 2000) and that had previously been validated for use in determining the body composition of *M. rutilus* (Stevenson and van Tets, 2008). Up to 16 bones were scanned at one time, and each was individually measured using the PIXImus2 software. The whole bone was used for each scan, and each bone was placed in the same position and orientation on the dual-energy X-ray apparatus scanning surface.

I used SPSS (version 14.0; SPSS Inc., Chicago, Illinois) statistical software to analyze the data. Multivariate analysis of variance (MANOVA) with Tukey's

honestly significant difference (HSD) post hoc test was used to determine whether there was an effect of season, sex, or the interaction term on the BMDs of the femur and humerus. I also used MANOVA to test for within-sex differences. Multiple regression analysis with stepwise variable entry was used to determine whether the independent variables of body mass, photoperiod, or body length explained a significant portion of the variability in the BMD of long bones. Mass and photoperiod were both included as entered variables because long-bone density changes proportionally with weight-bearing activity and photoperiod is strongly correlated with activity for *M. rutilus* (Stebbins, 1974; Tavernier *et al.*, 2004; West, 1977). Body length was included as a test variable as an index of frame size to control for the effect of variation in body size independently of body mass. Both body-mass and body-length measurements were log transformed to eliminate allometric effects. Animals were captured and treated following procedures approved by the American Society of Mammalogists (Gannon *et al.*, 2007), and all procedures were approved by the University of Alaska Anchorage Institutional Animal Care and Use Committee (IACUC), the Chugach State Park Service, and the Alaska Department of Fish and Game.

6.4 Results

Voles captured in winter were found in pairs or groups, consistent with the midwinter aggregation described by West (1977), and the expected seasonal change in body mass was observed (Table 6.1). Changes in the BMDs of the weight bearing bones of adult voles followed a seasonal pattern characterized by a rapid increase in spring, peak levels in early summer, and a gradual, continual decrease from highest values in early summer to late winter (Fig. 6.1). The mean bone density was significantly higher in spring and early summer than in fall and winter seasons for both bones (MANOVA with Tukey's HSD test, $p < 0.05$; $F = 13.65$, $df = 94$, $p < 0.001$ for femurs; $F = 11.88$, $df = 94$, $p < 0.001$ for humeri according to univariate tests over 6 seasons; Fig. 6.2), and minimum and

maximum bone densities also appeared to follow this trend (Fig. 6.1). Mass and photoperiod explained 46.2% and 45.7% of the total variation in BMDs in femurs and humeri, respectively (Table 6.2), with mass as the stronger predictor for femurs (body length was excluded from the model).

There were no between-sex differences in BMD under either the six season or collapsed four season model, possibly still due to small sample sizes in certain seasons. As an additional measure of the degree of seasonal change for each sex, I used a MANOVA for basic within-sex comparisons to determine whether a potential trend could be observed under the four season model. The test showed that although both sexes individually exhibited significant seasonal differences, males may undergo a slightly higher degree of seasonal variation. For males, BMDs of femurs were significantly lower in winter than in fall ($p < 0.05$), with levels in both of these seasons significantly lower than in spring and summer (Table 6.3). Females may undergo a slightly weaker seasonal change, because their BMD values in fall, winter, and spring did not differ significantly, although mean seasonal values appeared to follow the general seasonal trends of the population as a whole. Interestingly, the mean BMDs of both bones from each sex were highest in early summer, with mean values nearly identical. However, the mean BMD values of males were slightly higher than those of females in spring and late summer seasons, whereas females retained slightly higher BMDs than males in winter.

6.5 Discussion

I observed a pattern of seasonal change in BMD in a population of northern red-backed voles in Alaska that was characterized by dramatic increases in bone density during spring, peak values in early summer, and steady decreases in these values from early summer to late winter (Figs. 6.1 and 6.2). This pattern is likely to be related to the voles' seasonal changes in activity and breeding condition because BMDs of both bones were correlated with body mass and photoperiod

(an indirect indicator of activity). Other seasonal factors also may have contributed to these changes in BMD (*e.g.*, changes in hormone levels, appetite, food selection, life-history stage, energy and mineral availability in the environment, and vitamin D levels). However, the strong correspondence of activity and body mass with BMD (Table 6.2) is consistent with Wolff's law (Wolff 1892) and other, more recent studies linking bone remodeling to weight bearing activity (Blouin *et al.*, 2007; Resnick, 1988). When weight bearing activity is low, whether due to low body mass, low activity, or both, BMD is low. When weight-bearing activity is high, whether due to high body mass, high activity, or both, BMD is high. The combination of these factors appears to accelerate bone mineral depletion and osteopenia from late summer to late winter, and repletion in spring and early summer.

The observed pattern of change in bone density appears to be independent of age-related effects, such as the replacement of older adults dying off in summer or fall with younger adults entering the data pool. All animals in the study were of adult age. Body length (an additional indicator of age within the adult group) was excluded by the model and was therefore not a good predictor of bone density (Table 6.2). The observed pattern also appeared to be independent of any significant sex effects, but a larger sample size in certain seasons could prove otherwise. In high latitude regions, winter is a period of reduced activity, body mass (Table 6.1), sunlight exposure, vitamin D synthesis (Hickie *et al.*, 1982), and gonadal steroid synthesis in arvicoline rodents. Summer is the reverse. The ability to synthesize vitamin D or produce gonadal steroids in winter is likely to be particularly limited, as low photoperiod is combined with the subnivean lifestyle to further reduce exposure to sunlight. Mass may also be tied to photoperiod in this species because I have observed significant reductions in the body mass of subjects placed on manipulated photoperiods (switched from long days to short days) relative to control animals.

The positive increases in the BMDs of overwintered adults in late March that I observed just after the spring equinox (but before the onset of spring reproduction in April, Fig. 6.1), may mark the beginning of internal physiological changes in voles that eventually lead to increased body mass, activity, reproductive hormone levels, and BMD. Bones increased in density throughout spring, presumably because of the photoperiod linked increases in weight bearing exercise and gonadal steroid levels. When day length decreased between summer and fall, it is likely that BMD was affected by reduced activity levels associated with reductions in forage quality, ambient temperature, appetite, body mass, and reductions in the concentrations of gonadal steroids. As winter progressed and heavy snow and ice cover became established, BMD reached its lowest point (Fig. 6.1; Table 6.3), presumably as a result of further reductions in movement (Stebbins, 1974) and the occurrence of communal nesting (West, 1977).

Although the observed pattern of bone remodeling in *M. rutilus* occurred independent of sex effects, additional within sex comparisons revealed that only males showed a significant difference in femur BMD between fall and winter (MANOVA with Tukey's HSD, $p < 0.05$; Table 6.3). Males showed rapid increases in testis mass in spring (Nay *et al.*, 2007; see also Sealander, 1967; Whitney, 1976), which could contribute to intensified mate guarding or searching for females, along with greater home range. Males that are more robust (*i.e.*, have higher BMDs) and can more quickly assimilate minerals into bone matrix after winter may have an advantage over less robust males in mate guarding, female mate choice, and copulation success. Testosterone also may have a stronger effect on males' bones than estrogens and progestogens do on females' bones. The potential trend toward males having a slightly higher degree of change in BMDs than females in spring (Table 6.3) also may indicate a greater increase in activity of males, as is found in other arvicoline rodent species (Schmidt *et al.*, 2002). Interestingly, male and female voles had very similar bone mineral stores in early summer, which reflected their highest seasonal level. This may indicate that

mineral availability is not limited in this season and that the environment allows for maximum BMDs in both sexes (Table 6.3). By late summer, however, the BMDs of females appeared to be reduced in comparison to males, possibly an effect of a taxing reproductive season for this sex. This comparison of the degree of change within each sex revealed a potential trend toward bones of males having lower BMDs than bones of females in winter seasons. This may convey some sexual dimorphism in activity or food selection, but could also be linked to this and other species' ability to breed in winter (Hansson, 1984; Khlebnikov, 1970; Millar, 2001; Moffatt *et al.*, 1993; Whitney, 1976). Winter breeding does not occur in all years, and no winter breeding was observed in Chugach State Park populations between 2004 and 2007. However, because female arvicoline rodents can be induced into ovulation by copulation, they may be more constrained to maintain bone mineral stores in winter than males. Contact or copulation with a reproductively viable male is a rare but possible occurrence for female *M. rutilus* and other high-latitude arvicoline rodents (Hansson, 1984; Khlebnikov, 1970; Prendergast *et al.*, 2001), and this might induce ovulation or result in pregnancy, as it does in other species of Myodes (Clulow and Mallory, 1970; Odberg, 1984).

In summary, the northern red-backed vole, a nonhibernating, high latitude arvicoline rodent, does not maintain constant, yearlong bone mineral stores. Voles show rapid increases in BMD of long bones in spring, peak levels in early summer, and a gradual but continual decrease in BMD from early summer to late winter. These changes correspond with seasonal changes in body mass, activity, and hormones levels, although they also may be affected by forage quality, mineral and nutrient availability, and other factors.

The overall pattern of bone remodeling in *M. rutilus* undoubtedly reflects this species' overwintering strategy and life history. As a result, bone remodeling in *M. rutilus* provides a basis for comparative studies with other small high-latitude mammals, both nonhibernating and hibernating. The late season reduction and

rapid spring increase in bone density exhibited by free-living voles may, at some level, be useful in obtaining a better understanding of human osteoporosis.

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6.7 References

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6.8 Tables

Table 6.1 Seasonal Values for Body Mass of Adult *Myodes rutilus* in Southcentral Alaska. Mass undergoes seasonal change, and is a significant predictor of bone mineral density (Table 6.2), along with photoperiod (an indirect measure of activity). Combined data from 2004-2006 are shown, and means ± SE of body mass (g) are presented.

Season	$n = 97$	All voles	$n = 52$	Males	$n = 45$	Females
Spring	21	20.1 ± 0.7	12	20.8 ± 0.9	9	19.2 ± 0.9
Summer	21	26.4 ± 0.9	8	25.3 ± 1.5	13	27.1 ± 1.1
Early	14	27.0 ± 1.0	6	25.9 ± 1.8	8	27.9 ± 1.3
Late	7	25.2 ± 1.5	2	23.5 ± 3.1	5	25.9 ± 1.8
Fall	35	17.1 ± 0.3	20	17.4 ± 0.4	15	16.7 ± 0.4
Winter	20	13.4 ± 0.4	12	13.5 ± 0.4	8	13.2 ± 0.8
Early	14	13.4 ± 0.4	8	13.6 ± 0.5	6	13.0 ± 0.7
Late	6	13.4 ± 0.9	4	13.2 ± 0.8	2	13.7 ± 2.8

Table 6.2 Predictive Models for Femur and Humerus Bone Mineral Density (BMD) in *Myodes rutilus*. Multiple regression models utilize the independent variables of body mass and photoperiod to explain a significant amount of the variation in femur and humerus BMDs (46.2% and 45.7%, respectively). Body mass influences the density of weight-bearing bones, and thus, it is directly proportional to bone strength. Photoperiod is an indirect measure of activity (Stebbins, 1974; Tavernier *et al.*, 2004) and also helps to explain the variation in long bone density. Body length, an additional measure of age within the adult age class, was excluded by both models.

Multiple regression	*d.f.*	R^2	*F*	*P*-value	Include in model?	Unstandardized coefficient (*B*)
Femur BMD model	96	0.462	40.326	<0.001		
Entered variables						
Log body mass				0.001	Yes	0.024
Photoperiod				0.011	Yes	<0.001
Log body length				0.869	No	
Constant				0.155		0.010
Humerus BMD model	96	0.457	39.632	<0.001		
Entered variables						
Log body mass				0.030	Yes	0.015
Photoperiod				0.030	Yes	<0.001
Log body length				0.363	No	
Constant				0.001		0.017

Table 6.3 Within-Sex Comparisons of the Effect of Season on the Bone Mineral Density (BMD) of *Myodes rutilus*. Seasonal means ± *SE* of BMD are presented for male and female voles in different seasons. A multivariate analysis of variance (MANOVA) of the mean values for spring, summer, fall, and winter indicated a significant effect of season on bone density for both the femur and humerus ($p < 0.05$). Within each sex and for each bone, mean BMD values in each season that are represented by a shared letter are not significantly different ($p > 0.05$); femur and humerus means were never compared with one another, and males are not compared with females in this table. Within each sex, Tukey's HSD tests showed that mean bone densities were lower in fall and winter seasons than in spring and early summer seasons ($p < 0.05$). However, males appear to undergo a more pronounced seasonal change than females. The femur BMD of males was significantly lower in winter than in fall, with spring values well above both fall and winter for both bones. The spring, fall, and winter BMDs of female long bones were not significantly different. Although there were no significant between-sex differences according to the original MANOVA, there are apparent trends towards males having higher BMDs in spring and late summer seasons and towards females having higher BMDs in winter seasons.

	Males n = 52			Females n = 47		
Season	n	Femur BMD	Humerus BMD	n	Femur BMD	Humerus BMD
Spring	12	.050 ± .002 a	.044 ± .001 a	9	.045 ± .002 ab	.042 ± .001 ab
Summer	8	.052 ± .002 a	.044 ± .002 a	13	.049 ± .002 a	.044 ± .001 a
Early	6	[illegible]	[illegible]	8	[illegible]	[illegible]
Late	2	[illegible]	[illegible]	5	[illegible]	[illegible]
Fall	20	.043 ± .001 b	.039 ± .001 b	15	.042 ± .001 b	.038 ± .001 b
Winter	12	.038 ± .001 c	.036 ± .000 b	8	.041 ± .001 b	.038 ± .000 b
Early	8	[illegible]	[illegible]	6	[illegible]	[illegible]
Late	4	[illegible]	[illegible]	2	[illegible]	[illegible]

6.9 Figures

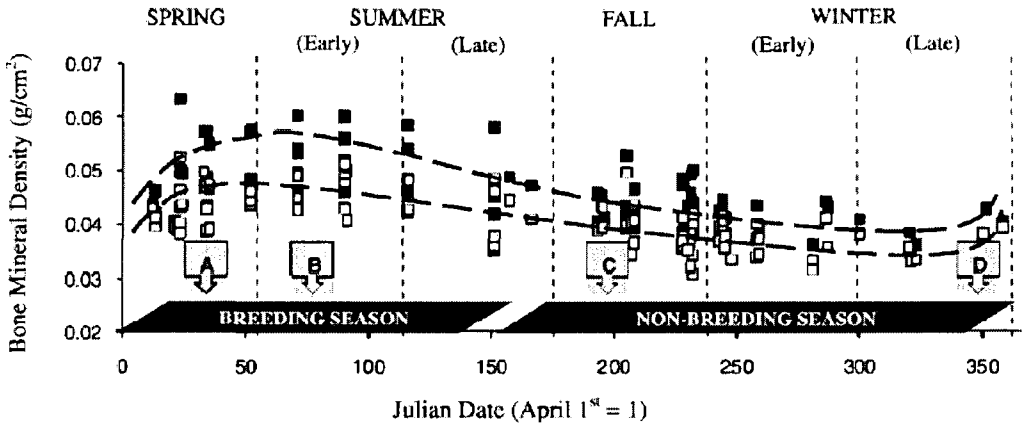

Figure 6.1 Annual Pattern of Change in Bone Density of Femur (shaded squares) and Humerus (unshaded squares) in a Population of *Myodes rutilus* in Alaska. BMD is plotted against Julian date, and data from November 2004 – January 2007 are included. Numbers on the X-axis correspond to calendar dates beginning with the start of spring: 1 = April 1st (start of spring), 100 = July 8th (mid-summer), 200 = October 16th (fall), and 300 = January 24th (mid-winter). A clear seasonal pattern of bone remodeling is shown in adult voles: an increase in BMD from the end of winter through spring, peak values in early summer, and gradual but continual decreases in these values to late winter (osteoporosis). Periods of reproductive activity for this species are indicated at the bottom of the graph, and dashed vertical lines indicate the defined seasons. General characteristics of this species life-cycle are indicated by letters near the bottom of the graph: A = First occurrence of parturition, B = First occurrence of young-of-year reaching adulthood, C = End of lactation and weaning, D = Spring equinox.

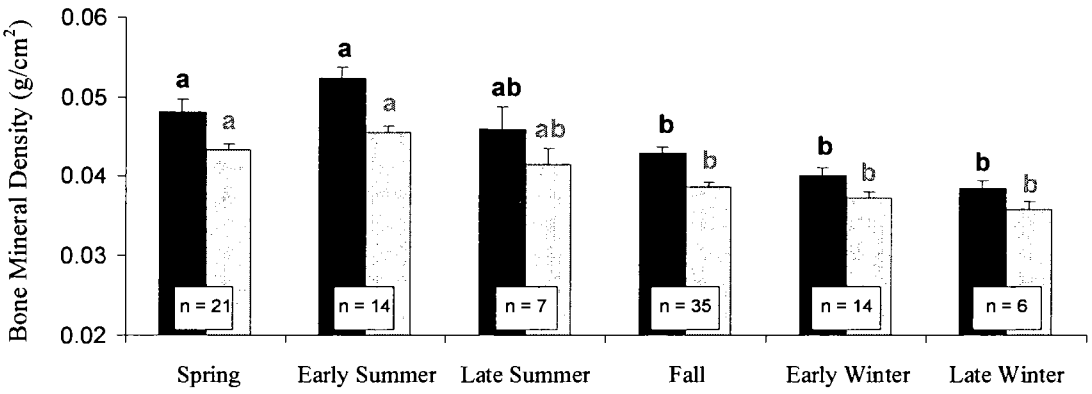

Figure 6.2 Seasonal Changes in Long Bone Mineral Density (BMD) of Femur (black bars) and Humerus (gray bars) Under a Six-Season Model. Mean values represented by bars that share the same letter in the same color are not significantly different ($p > 0.05$). BMD was significantly lower in fall and winter than in spring and summer seasons. Bone densities rapidly increase from late winter to early summer, but then undergo osteopenia from late summer to late winter.

Chapter 7:
Effect of Overwintering on Body Mass and Bone Mineral Density in Two Hibernating Mammals: Arctic Ground Squirrels (*Spermophilus parryii*) andAmerican Black Bears (*Ursus americanus*)

7.1 Abstract

Humans and other mammals undergoing prolonged periods of inactivity suffer a severe loss in bone mineral density (BMD) due to an absence of a mechanical loading on the skeleton. Arctic ground squirrels (*Spermophilus parryii*) and American black bears (*Ursus americanus*) each undergo a 5-7 month period of hibernation during which time little or no mechanical stress is placed on the bones. Winter disuse osteoporosis could be occuring in these animals, but reductions in their blood flow, metabolic rate, and body temperature make this uncertain. Therefore, the aim of this study was to measure the effect of hibernation duration and ambient temperature on the body mass and bone density of arctic ground squirrel and black bear and to compare it with the effect of overwintering on body mass and bone density in nonhibernating redbacked voles, which have both previously been shown to decrease BMD in winter. Dual-energy X-ray absorptiometry (DXA) was used to measure the BMDs in all three species. During hibernation, the ground squirrels showed a significant decrease in body mass from 825 ± 29 g to 644 ± 28 g (paired t-test, $n = 34$, $p < 0.001$), but maintained their femoral BMD throughout the hibernation period (initial BMD 0.1966 ± 0059g/cm^2, final BMD 0.1951 ± 0.0058 g/cm^2; paired t-test, $p = 0.574$). Ambient temperature (+2 °C vs. -10 °C) and duration of hibernation (range of 45 to 183 days) had no effect upon overall change in BMD in arctic ground squirrels (ANCOVA, corrected model $F_{[1,31]} = 0.660$, $p = 0.768$), but both factors were related to the overall change in body mass (ANCOVA, corrected model $F_{[1,31]} = 16.339$, $p < 0.001$; temperature $F_{[1,31]} = 88.120$, $p < 0.001$; duration of hibernation

$F_{[1,31]} = 5.182$, $p = 0.001$). Black bears' ($n = 4$) body mass declined during their hibernation of 132 ± 6 d from 76.000 ± 7.062 kg to 63.175 ± 6.635 kg ($p = 0.003$). Bone mineral density of the phalynx diaphysis showed little overall change, but densities of diaphysis midpoints decreased slightly in all bears (-0.0522 ± 0.0195 g/cm^2; paired t-test, $p = 0.075$) and were weakly correlated with date of emergence (simple regression, $p = 0.048$, $R^2 = 0.91$). The relationship between date of emergence and diaphysis midpoint BMD in black bears, but not squirrels, could be related to differences in metabolic state during hibernation between the two species. It is more likely, however, that it is related to a slower or more variable rate of emergence in bears relative to the smaller arctic ground squirrels, which either emerged quickly or were euthanized prior to emergence. That BMD did not change in the true hibernator (*S. parryii*), decreased slightly in the weak hibernator (*U. americanus*), and underwent a seasonal osteopenia in the non-hibernator (*M. rutilus*) suggests that the extent of winter osteopenia is also reduced, or even in *S. parryii* rendered so small as to be undetectable.

7.2 Introduction

In humans and other mammals, reductions in bone density can result from restricted movement and a lack of mechanical stress on bones ('disuse osteoporosis') (Resnick, 1988; Blouin *et al.*, 2007), prolonged spaceflight (a special case of disuse osteoporosis) (Biryukov and Krasnykh, 1970; Collet *et al.*, 1997; LeBlanc *et al.*, 2000; Milstead *et al.*, 2004), changes in diet and mineral uptake (Gennari, 2001; Rodriguez-Martinez and Garcia-Cohen, 2002; Demigne *et al.*, 2006), reductions in baseline concentrations of gonadal steroids and growth hormone (GH) (Christmas *et al.*, 2002), or seasonal fluctuations in body mass and photoperiod (Stevenson *et al.*, 2009b). In the case of prolonged bed rest, bone formation rates decrease, osteoblast surface decreases, and osteoclast surface increases (Arnaud *et al.*, 1992; Zerwekh *et al.*, 1998). Hibernators, although in a

state of torpor, also place virtually no mechanical stress on their bones for months at a time, suggesting that they could also undergo osteopenia or osteoporosis.

I recently measured seasonal changes in bone density of a free-living, nonhibernating rodent in Alaska, the northern red-backed vole (*Myodes rutilus*) and observed a pattern of osteopenia from summer to late winter (Chon *et al.*, 2008; Stevenson *et al.*, 2009a). Changes in bone density were most strongly correlated with changes in body mass and photoperiod, an indirect measure of activity (Stebbins, 1974; Tavernier *et al.*, 2004), and were presumably also linked to sex-steroid levels. Some mammals in arctic and subarctic Alaska, however, hibernate to survive extended winters, which are characterized by very cold temperatures and reduced food availability. Arctic ground squirrels (*Spermophilus parryii*) and American black bears (*Ursus americanus*) undergo a 5-7 month period of hibernation during which body temperature, metabolic rate, and heart rate are reduced. No food is consumed during hibernation, and animals minimally urinate and defecate. As little or no mechanical stress is placed on the bones at this time, the potential for winter disuse osteoporosis exists. The reductions in these species' blood flow, metabolic rate, and body temperature that are associated with hibernation (Boyer and Barnes 1999; Barnes and Buck, 2000) may, however, concurrently reduce the need for non-bone Ca^{2+} and inhibit bone resorption. It is not clear what effect, if any, hibernation might have on BMD in these two species.

The hibernation processes in arctic ground squirrels and black bears differ in their character (i.e., true hibernation vs. winter torpor). Black bears maintain relatively higher heart rates (HR), resting metabolic rates (RMR), and body temperatures ($T_b \sim 30^{\circ}C$) during their "winter sleep", and females often enter dens in mid-gestation and emerge with cubs in spring. Their relatively moderate body temperatures and metabolic rates during hibernation may allow for increased function of osteoblasts, osteoclasts, hormones, proteins, gene transcription, and growth factors. The large body size of *U. americanus* may also slow their rate of

warming during emergence in spring. In contrast, arctic ground squirrels hibernate in a drastically reduced metabolic state with a HR of 3 to 5 beats per minute. Ground squirrels often maintain internal T_b near the blood freezing point (-0.6°C), and some individuals have been known to supercool their abdomen to -2.9°C (Barnes, 1989). Hibernating *S. parryii* also undergo regular and reoccurring bouts of arousal every 6-14 days (depending on ambient temperature), at which time metabolism, HR, and T_b rise to normal levels for about 24h (Barnes and Buck, 2000). These bouts are energetically expensive and may be requirements for sleep (Daan *et al.*, 1991; Barnes *et al.*, 1993). The inverse relationship between ambient temperature (T_a) and length of arousals implies that animals in colder dens will have a higher frequency, and thus, more total arousals during winter. If bone resorption occurs primarily during arousal, animals hibernating at colder temperatures should have lower BMDs than those hibernating at higher temperatures. As, however, *S. parryii* spends the majority of the winter presumably maintaining temperatures low enough to severely reduce protein function and gene transcription, they may undergo very little bone resorption at all, regardless of the temperature in their hibernacula.

The effect of hibernation (or estivation) on bone stores appears to vary by species, metabolic state, and duration of torpor. Not all animals hibernate in the same manner, and there is not a consistent pattern of bone loss or defense against bone loss across taxa. For example, the green-striped burrowing frog, *Cyclorana albuguttata*, is thought to defend bone integrity and bone size during its three- to nine-month period of inactivity (Hudson *et al.*, 2004), while significant bone loss occurs during hibernation in the golden hamster (Steinberg *et al.*, 1979; Steinberg *et al.*, 1981; Steinberg *et al.*, 1986) and the 13-lined ground squirrel (*Citellus tridecemlineatus*) (Haller and Zimny, 1977). The little brown bat, *Myotis lucifugus lucifugus*, lacks bone-forming osteoblasts during hibernation, with all new bone formation occurring after arousal (Kwiecinski *et al.*, 1987). In free-living black bears bone resorption remains elevated over the entire hibernation

period (relative to the pre-hibernation period), but osteoblastic bone formation is not impaired by hibernation and is rapidly accelerated during remobilization (Donahue *et al.*, 2003b). Additionaly, bone volume, mineral content, porosity, and strength do not appear to be adversely affected by annual periods of disuse in this species (Harvey and Donahue, 2003; Pardy *et al.*, 2004; Harvey *et al.*, 2005; Donahue *et al.*, 2006). Most of these studies are, however, limited by the lack of repeated measurements of bone density over the course of hibernation. The aim of this study was to use non-lethal imaging technology to make repeated measures of the pre- and post-hibernation state of bone in two hibernating species, the arctic ground squirrel (*S. parryii*) and the American black bear (*U. americanus*), and to compare the effects of hibernation on the body mass and BMD of these two species with the reported effects on *M. rutilus* – a nonhibernating rodent species whose range overlaps theirs.

7.3 Methods

7.3.1 Arctic Ground Squirrels

Forty-three arctic ground squirrels, *S. parryii*, were live-captured above the Arctic Circle, north of the Brooks Mountain Range in Alaska and adjacent to the Dalton Highway. They were transported to the University of Alaska Fairbanks (UAF) and housed in individual cages in UAF's animal quarters. Hibernation was induced in experimental animals by altering the photoperiod to constant dark and setting T_a to either 2°C (n = 16) or 10 °C (n = 17). Some ground squirrels remained euthermic (did not hibernate, n = 7) and were used as a control group at 2°C. These euthermic animals were analyzed in a similar fashion as treatment subjects and were kept in identical conditions (except that they were given *ad lib* access to food and water).

Squirrels were weighed to the nearest 0.1g, and femoral bone densities were measured using a PIXImus2 Dual-energy X-ray absorptiometry (DXA) apparatus (GE/Lunar) and accompanying Lunar PIXImus2 2.00 software. DXA accurately

and non-destructively measures bone and tissue content in live, anesthetized animals (Nagy and Claire, 2000; Felicetti *et al.*, 2003; Stevenson and van Tets, 2008), and has been used to show seasonal changes in the BMDs of red squirrels (*Sciurus Vulgaris*) (Garriga *et al.*, 2004), northern red-backed voles (*M. rutilus*) (Chon *et al.*, 2008; Stevenson *et al.*, 2009a), and black bears (*U. americanus*) (Pardy *et al.*, 2004). DXA is a non-lethal technique that permits serial measurements of the same individual over time for repeated-measures analyses. For this study, DXA quality control statistics were set to allow no more than 0.2% error during calibration using a standard provided by the manufacturer.

Two days into hibernation, the ground squirrels were briefly aroused, anesthetized (3-5% isoflurane, vaporizer), and placed in ventral recumbency over the DXA apparatus platform (Fig. 7.1a). The hind left leg was stretched to obtain a consistent position to encompass the entire femur, and the "region of interest" function of the DXA software was used to limit data to the target bone. The second BMD measurements were made after squirrels were euthanized with an overdose of sodium pentobarbital (1 ml/kg). The whole left hind leg was removed and frozen at -20°C and then later thawed for analysis. Some animals were allowed to complete hibernation before being euthanized. If a ground squirrel was found to be awake for three consecutive days, it was regarded as having emerged from hibernation. Post-hibernation scans were obtained by thawing squirrel femurs to room temperature, placing them in a consistent position on the DXA platform, and scanning them with the DXA apparatus. All procedures involving arctic ground squirrels were approved by the UAF Institutional Animal Care and Use Committee (IACUC, protocol 06-40).

7.3.2 Black Bears

Four black bears, *U. americanus*, were obtained from the Alaska Department of Fish and Game for research purposes at the University of Alaska Fairbanks. In November and prior to hibernation, bears were immobilized with Thelazol (and

Ketamine, when needed). The right paw of each bear was placed on the DXA platform in a consistent position (Fig. 7.1b) to obtain a scan of the second middle phalanx. The diaphysis of the target bone was isolated using the same DXA apparatus and software as with arctic ground squirrels, and BMD measurements of both the entire diaphysis and the diaphysis midpoint (20 center pixels where tensile strength is weakest) were obtained. The bears were then moved to enclosed outdoor winter dens where they were monitored by video camera and remote biological equipment. Time of emergence was determined both by monitoring body temperature and by a general evaluation of the bears' behavior. Criteria for defining a bear as emerged required that it had a body temperature of 36°C, bumped the door to the den, and began emerging from the den. All three criteria had to be met for the bear to be defined as emerged. The first three bears to emerge did so on three consecutive days beginning on the 125th day of hibernation. The final bear emerged approximately 3.5 weeks later at day 150. The body temperature of this bear was the same as the others upon emergence and did not appear to be any more active. Within 24h of emergence, each bear was anesthetized and subsequently euthanized (1ml/kg sodium pentobarbital). The right paw was then amputated from the bear and frozen at -20°C for post-hibernation DXA analysis, where the paw was placed on the platform in an identical manner to the pre-hibernation scan. All procedures involving black bears were approved by the UAF IACUC (protocol 05-55 and 05-56).

7.4 Results

During hibernation, arctic ground squirrels, *S. parryii* (n=34), showed a significant decrease in body mass (paired t-test $t_{33} = 13.72$, $p < 0.001$; Fig. 7.2a). Ambient temperature and duration of hibernation each had an effect upon overall change in body mass (ANCOVA corrected model $F_{[1,31]} = 16.34$, $p < 0.001$; Covariates: temperature $F_{[1,31]} = 88.120$, $p < 0.001$, hibernation duration $F_{[1,31]} = 5.18$, $p = 0.001$, Fig. 7.2a). Ground squirrels did, however, maintain femoral bone

mineral stores throughout hibernation from its onset (0.1966 ± 0059g/cm^2) to its conclusion (0.1951 ± 0.0058 g/cm^2) (paired t-test $t_{33} = 0.61$, $p = 0.574$, Fig.7.2b). There was no effect of either ambient temperature (+2 °C vs. -10 °C) or duration of hibernation (45 to 183 days) on overall change in BMD (ANCOVA corrected model $F_{[1,31]} = 0.66$, $p = 0.768$; Covariates: temperature and hibernation duration, not significant; Fig. 7.2b).

The captive black bears, *U. americanus* (n = 4), also showed a significant decrease in body mass (-12.23kg ± 1.42 during hibernation ($t_3 = 8.618$, $p = 0.003$, Fig. 7.3a) over 132d ± 6 at an average rate of -0.123 kg/d. The phalynx diaphysis BMD showed little overall change, but each of the diaphysis midpoint BMD values were at least slightly (although not significantly) reduced in each bear (-0.0522 ± 0.0195 g/cm^2; paired t-test $t_3 = 2.674$, $p = 0.075$), much of this due to the final bear to emerge (Figs. 7.3b and 7.3c). This bear had the longest hibernation duration and declined in BMD of the diaphysis midpoint relative to the other three bears that had emerged at least three weeks before (Simple regression, corrected model $F_{[1,3]} = 19.289$, $p = 0.048$, $R^2 = 0.91$).

7.5 Discussion

The ability of these two arctic/subarctic hibernating species to conserve bone mineral stores during winter despite significant body mass loss is in stark contrast to the seasonal osteopenia observed in the northern red-backed vole (*M. rutilus*), whose range overlaps both *S. parryii* and *U. americanus*, but who maintains a constant T_b and active metabolism during winter (Stevenson *et al.*, 2009a). These voles undergo drastic seasonal changes in both their behavior and physiology. They form communal nests in winter and severely restrict activity, home range, and movement (Stebbins, 1974; West, 1977). They also experience severe decreases in body mass, internal organ mass, and reproductive mass (Sealander, 1967; Zuercher *et al.*, 1999; Nay *et al.*, 2007; Stevenson *et al.*, 2009b). The bone density of *M. rutilus* decreases in from early summer to late winter before rapidly

increasing again in spring. This is due, in part, to the effect of reductions in the mechanical load on the skeleton (*i.e.*, lower body mass), weight bearing exercise, and baseline reproductive hormone levels (Stevenson *et al.*, 2009a). If viewed in terms of generalized activity or metabolic activity only, this is the opposite of what might be expected: the winter-active mammal undergoes a reduction in bone density while the winter-inactive mammals maintain theirs. This apparent discrepancy is most likely explained by their different approaches to overwintering.

The body mass of both *S. parryii* and *U. americanus* decreased significantly over hibernation (Figs. 7.2a and 7.2b), and there was a significant effect of ambient temperature on squirrel body mass. The body mass loss is most probably due to fatty acid metabolism and release of ketone bodies (Boyer and Barnes, 1999). The ground squirrels housed at -10°C lost more body mass than those housed at 2°C, and the effect became cumulatively greater over longer durations of hibernation (Fig. 7.2a). Ground squirrels that hibernate at colder temperatures are, therefore, likely to exit winter hibernacula and begin the mating season in poorer condition, with respect to body mass, than those which find or build warmer dens. Much of this can depend on habitat selection, as arctic ground squirrel burrows with shrubby vegetation accumulate more snow and have higher soil temperatures over winter than windswept sites in non-shrubby vegetation (Buck and Barnes 1999). Although free-living ground squirrels occupying colder burrows in this previous study were predicted to lose more body mass during hibernation than those in warmer burrows, changes in body condition over winter were not correlated with soil temperature – presumably due to confounding factors not related to the gradient between body and soil temperatures, such as use of food caches or differing thermal conductance of nests. These variables have been eliminated in the present study, and a significant effect of temperature on body mass was detected.

Bone mineral density was, however, generally conserved in both species (Figs. 7.2b and 7.3b). Squirrels are deep hibernators having extremely low body temperatures, heart rates, and metabolic rates (Barnes, 1989; Boyer and Barnes, 1999; Barnes and Buck, 2000). There was no observable BMD loss, suggesting that the processes normally involved in resorbing bone in mammals that exhibit no weight-bearing activity for prolonged periods of time are inhibited in the hibernating ground squirrel, *S. parryii*. This may be related to the extremely low body temperature of this species, which could severely reduce protein function, gene transcription, and molecular mechanisms common in disuse osteoporosis (reviewed by Takata and Yasui, 2001). It could also be a function of systematic hormones and growth factors involved in continuing bone formation to maintain bone mass in the absence of mechanical loading despite a cold body temperature.

That the arctic ground squirrels do not undergo any observable bone resorption during hibernation stands in stark contrast to prior studies on hibernating rodents at lower latitudes that show bone loss during prolonged periods of hibernation (Haller and Zimny, 1977; Steinberg *et al.*, 1979; Steinberg *et al.*, 1981; Steinberg *et al.*, 1986). Arctic ground squirrels may possess unique qualities that allow them to maintain bone density during a more prolonged and harsher winter environment relative to these other rodent species. Whether the hormonal and cellular functions that appear to protect bone stores in hibernating bears also act in arctic ground squirrels to conserve bone density is not known. The probability seems low due to their near-freezing body temperatures and very low HRs and RMRs. It is more likely that the extreme and prolonged lowering of T_b during hibernation in this species, along with its drastically lowered blood flow, metabolic processes, and enzyme activities (Barnes, 1989; Boyer and Barnes, 1999) reduce any other bone resorption below detectable limits. They may simply be employing a strategy of "slowing and cooling" so that blood flow nearly stops, enzyme activities are drastically low, and many normal metabolic

processes cannot entirely function - including those involved in the bone resorption response to a prolonged absence of mechanical stress on the skeleton.

The overall change in BMD of black bears was also not significant ($p = 0.075$), possibly due to the low sample size and/or only minimal decreases in the first three bears to emerge, which are likely to be normal if the last point is, indeed, an outlier (Fig. 7.3c). The data points representing BMDs of the last emerging bear contributed the most to the observed downward trend in diaphysis midpoint BMD. The most likely explanation of the observed pattern is that black bears undergo almost no bone loss during the first 125 days of winter sleep. The last data point, collected 3.5 weeks later, was either an outlier or began to decrease in bone density later into hibernation. Bears are more metabolically active than hibernating ground squirrels and have much higher body temperatures while overwintering (30°C vs. 0°C). The non-significant decrease in diaphysis midpoint BMD may have reflected this ($n = 4$, $p = 0.075$; Figs. 7.3b and 7.3c). Furthermore, bone resorption markers are known to increase during hibernation, suggesting high bone turnover during periods of inactivity (Donahue *et al.*, 2003a). The large body size of *U. americanus* increases the time required by this species to elevate T_b to the emergence level (36°C), and the metabolic and hormonal processes (including those involved in bone resorption and rapid change of bone markers) are likely to be already functional during this period.

Neither *S. parryii* nor *U. americanus* showed any striking decreases in BMD during hibernation despite a considerable loss in mass. Systemic hormones and growth factors may be involved in continuing bone formation to maintain bone mass in the absence of mechanical loading. For instance, there is some evidence that parathyroid hormone, prostaglandin, leptin, and insulin-like growth factor levels all vary between pre-hibernation, hibernation, and post-hibernation periods in *U. americanus* (Donahue *et al.*, 2006). Studies on rats (*Rattus norvegicus*) have also suggested a positive effect of leptin on bone maintenance after disuse (Martin *et al.*, 2005). Since bone mineral stores are defended during hibernation, these

factors and other molecular mechanisms are likely to contribute to bone preservation.

7.6 Conclusion

There was no detectable bone loss in the true hibernator, *S. parryii*, which spends most of the winter at a T_b below 0°C with its metabolic activity dramatically reduced. There was little or no detectable bone loss in *U. americanus*, which overwinters in a state of 'winter sleep' with a slightly reduced T_b and metabolism. A winter osteopenia exists in *M. rutilus*, a metabolically active mammal with a relatively constant T_b throughout the winter. It appears that hibernation itself, possibly combined with other bone defense mechanisms, inhibits bone resorption in high-latitude mammals.

7.7 Acknowledgements

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7.9 Figures

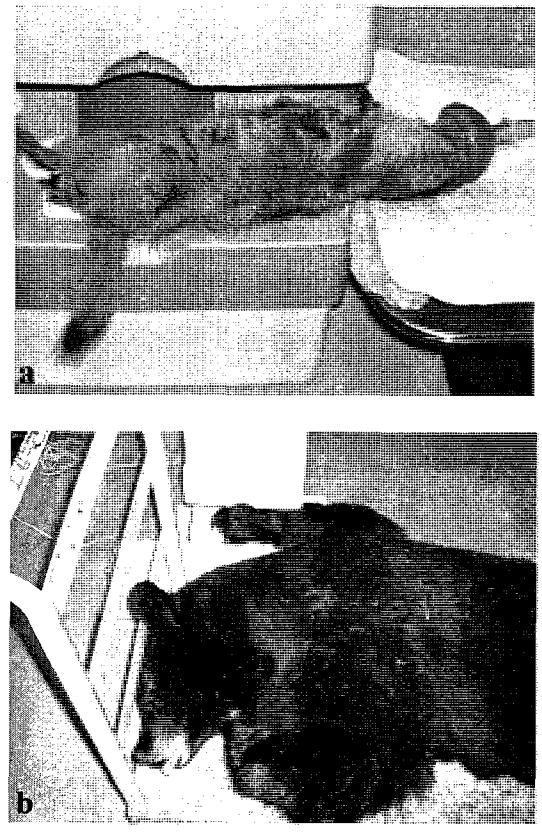


Figure 7.1 Dual Energy X-Ray Absorptiometry (DXA) Analysis of an Arctic Ground Squirrel (*Spermophilus parryii*) and an American Black Bear (*Ursus americanus*). DXA data and images were obtained from the femurs of arctic ground squirrels (*Spermophilus parryii*) (*a*) and the middle phalynxes of American black bears (*Ursus americanus*) (*b*).

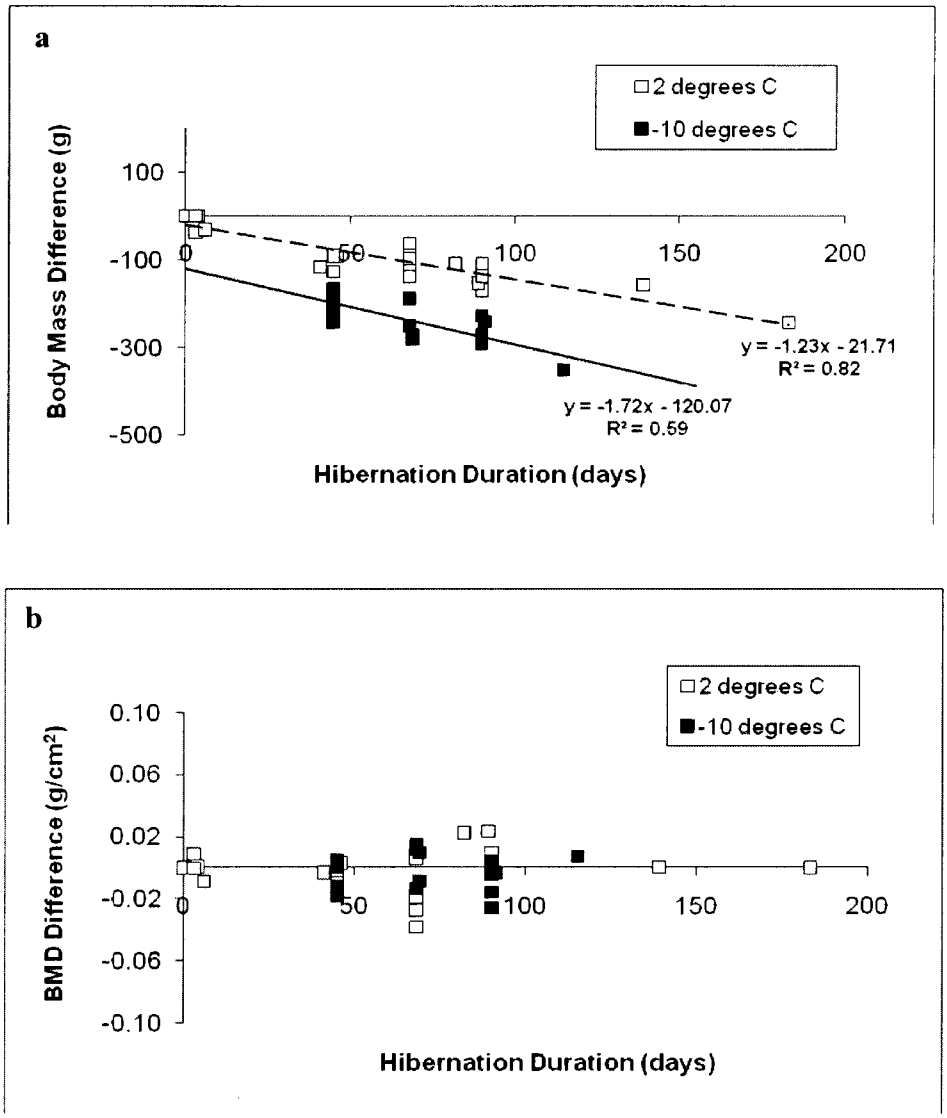


Figure 7.2 Effect of Hibernation Duration and Temperature on Body Mass and Femoral Bone Mineral Density (BMD) of Arctic Ground Squirrels (*Spermophilus parryii*). (*a*) Change in body mass and duration of hibernation were tightly correlated for *S. parryii.* There was also a significant ($p < 0.05$) effect of ambient temperature on body mass. Euthermic control animals did not decrease in mass (not shown). (*b*) There was no significant relationship between change in femoral BMD and either the duration of hibernation for *S. parryii* ($p > 0.05$) or ambient temperature ($p > 0.05$).

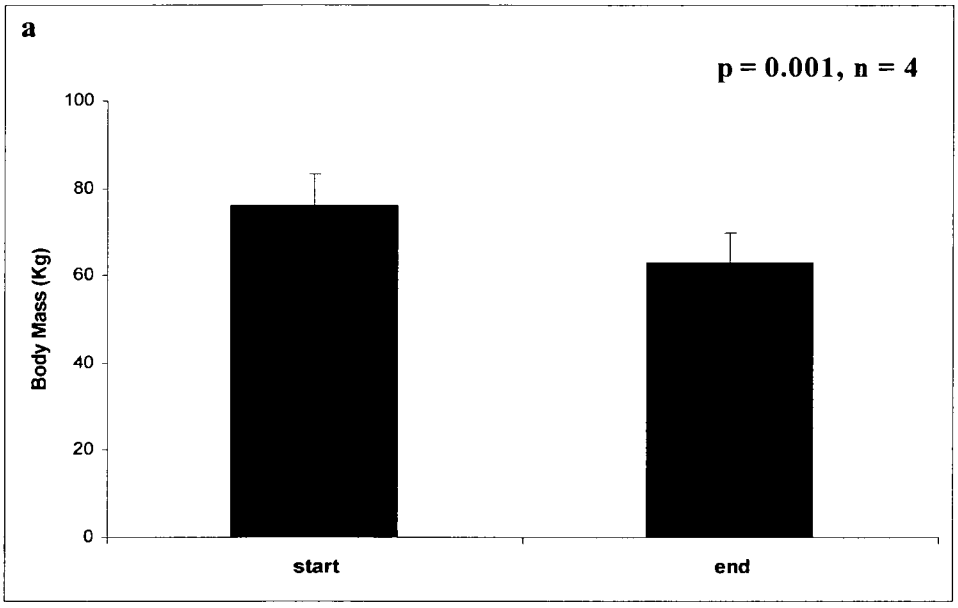
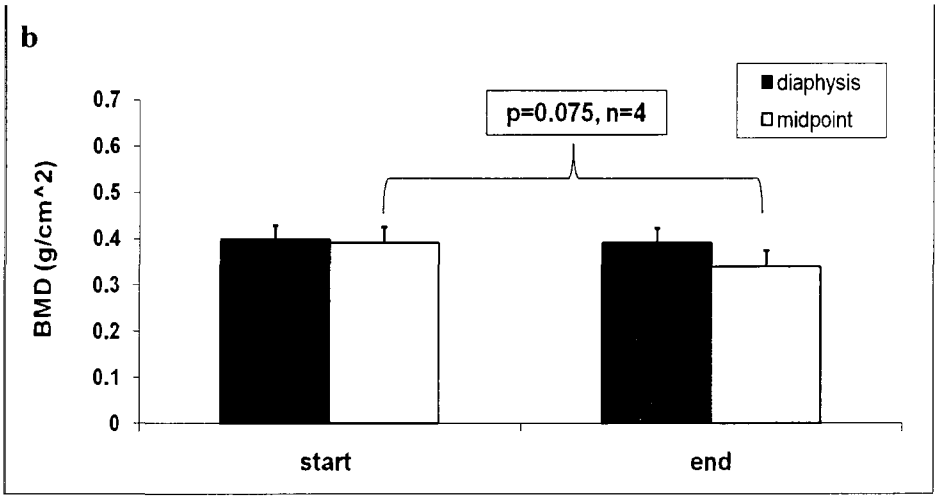


Figure 7.3 Effect of Hibernation Duration on Body Mass and Bone Mineral Density (BMD) of the Second Middle Phalynx of American Black Bears (*Ursus americanus*). (*a*) Mean body mass of bears (with standard error bars) representing pre- and post-hibernation values. Post-hibernation body mass was significantly lower (n = 4, paired samples t-test, p = 0.003). (*b*) Mean changes in BMD of phalynx diaphysis and diaphysis midpoint. Bars represent mean ΔBMD values, and error bars represent standard errors. Diaphysis BMD showed little overall change, but diaphysis midpoint BMD decreased, at least slightly, in each bear (paired t-test p = 0.075, non-significant). (*c*) Effect of hibernation duration on BMD. The first three bears to emerge produced data points that were tightly grouped. Either the last bear to emerge was an outlier or there was some effect of its longer hibernation period and later date of emergence on BMD (simple regression, p = 0.048, R^2 = 0.91). This individual showed severe declines in BMDs of both the diaphysis and the diaphysis midpoint relative to the other three bears that had emerged at least three weeks before.



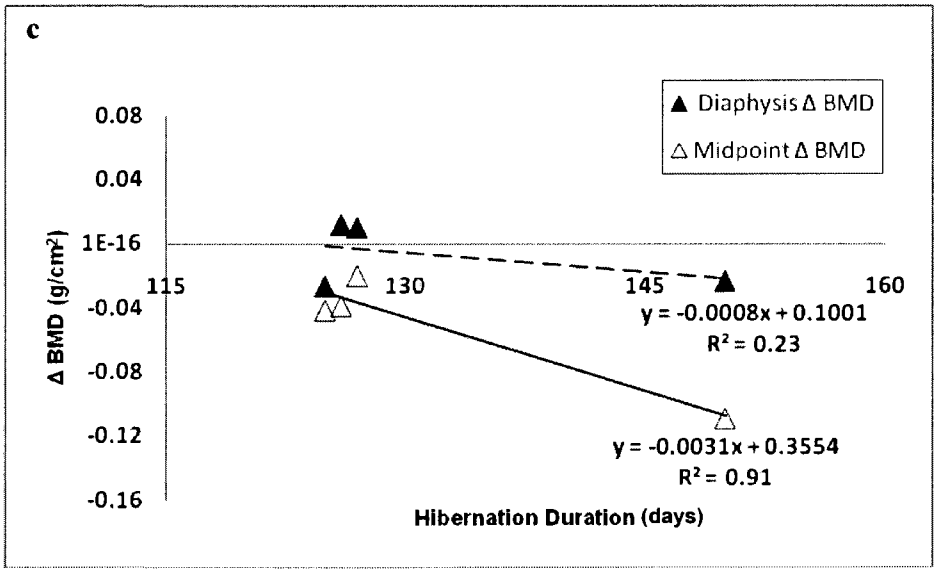


Figure 7.3 Continued…

Chapter 8:
Conclusions

8.1 Summary and Conclusions

Arvicoline rodents inhabit high-latitude environments and undergo pronounced seasonal changes in their physiology and behavior. They do not hibernate or migrate, and they remain active in winter within the subnivean space. As herbivores, they shape local plant communities (Schultz, 1964; Howe and Brown, 1999; Howe *et al.*, 2002). As prey items, voles and lemmings determine the distribution and densities of many predatory species (Maher, 1970; Wilson and Bromley, 2001; Gilg *et al.*, 2003; Hudson and Bjornstad, 2003). They undergo pronounced population cycles and numbers can vary by as many as two orders of magnitude between years (Erlinge *et al.*, 2000; Gilg, 2002; Turchin *et al.*, 2000, Gilg *et al.*, 2003; Framstad *et al.*, 1993). Their population numbers can affect the survival and reproductive fitness of secondary prey species (Gilg *et al.*, 2003; Maher, 1970; Wilson and Bromley, 2001; Sittler *et al.*, 2000). As a result, fluctuations in arvicolien rodent populations are of fundamental interest to ecologists working in arctic and sub-arctic ecosystems, and this has been a driving force behind the search for the mechanisms that underpin their population cycles. There is also increasing interest in how the physiology and ecology of voles and lemmings might be shaped by changes in climate.

Reproduction and lactation are the most energetically expensive processes undergone by mammals (Degen *et al.*, 2002, Liu *et al.*, 2003), so breeding usually occurs when all other costs are lowest. Arvicoline rodents may, however, effectively utilize microhabitats (Orrock and Pagels 2003) or acclimatize to their seasonal environment (McDevitt and Speakman, 1996, Kenagy and Pearson, 2000) to minimize thermoregulatory demands, investing excess available energy into reproduction during a traditionally non-breeding season. Proximate

(environmental) factors help animals align expensive breeding processes with predicted times of peak food availability. This is potentially problematic, however, as photoperiod is constant while other factors are variable.

Winter breeding is rare, but has occured in almost every arvicoline rodent species studied (reviewed in Hansson, 1984; Millar, 2001), although not in all individuals or all years. Seasonal and out-of-season breeding can significantly impact next-summer populations of voles and lemmings, and ecological work on population cycling in high-latitude rodents has been hindered by a lack of data on year-round data for breeding frequency, reproductive condition, and related factors (*e.g.*, body composition and field metabolic rate).

Arctic and subarctic climates are linked to food availability in arvicoline rodents. The occurrence of warmer temperatures in earlier seasons for several consecutive years could facilitate natural selection for animals that use predictive cues for peak food availability. Furthermore, a disruption in this hypothetical trend (*e.g.*, a very cold or wet year after many warmer seasons) could have deleterious consequences for vole and lemming populations (and their predators) that had undergone selection for individuals to thrive in warmer climates. This could result in increased cold exposure, starvation, increased pressure from predation, inhibition of breeding, or termination of pregnancies.

The predictability of vole and lemming cycles at high-latitudes might also be affected by environmental change. There are suggestions that the once strong and predictable population cycles of arvicoline rodents in Northern regions are already disappearing, with prolonged interruptions in cycles attributed to the effects of extrinsic factors, such as change in climate (Hörnfeldt *et al.*, 2005; Angerbjörn *et al.*, 2009). The decrease in cycle predictability could be related to decreases in predictability of food, which might limit population booms. Patterns of body composition are also important with respect to rodent cycling, as energy is allocated more toward either somatic growth or reproductive effort, depending on the phase of the population cycles (Norrdahl and Korpimäki, 2002). Should

climate change in a substantial and predictable manner in arctic and subarctic regions, however, there could be a positive or negative effect on arvicoline reproductive condition, energy stores, seasonal regulatory functions, survival, or annual fitness. The exact effect will be mediated by physiological mechanisms, and studying effects of season and environmental factors on the physiology of free-living and captive arvicoline rodents is therefore crucial. My overall aim, therefore, was to measure the effects of season and environmental variables on the reproductive axis, body composition, and energy expenditure of the northern red-backed vole (*Myodes rutilus*), an arvicoline rodent species that is common in Alaska and that is known to have bred in winter (Khlebnikov, 1970; Whitney, 1976; Hansson, 1984).

To achieve my overall aim, I first validated a dual-energy X-ray absorptiometry (DXA) apparatus for use in free-living *M. rutilus* to assess parameters of body condition (Chapter 2, Stevenson and van Tets, 2008). I showed that DXA can provide reliable data for both free-living and lab-raised rodents, and I derived predictive algorithms to calculate body composition values ($R^2 = 0.82$ to $R^2 = 0.98$ for all components, free-living and lab-raised groups combined). I concluded that DXA is capable of accurately measuring levels of fat, lean mass, body water, protein, and mineral, even in very lean rodents. This then allowed me to use DXA to determine body composition of voles in my various studies.

I next studied the seasonality of reproduction in photoperiod responsive and non-responsive *M. rutilus* in Alaska (Chapter 3; Nay et al., 2007; Stevenson *et al.*, 2009b). By combining trapping of free-living voles with captive studies, I was able to measure frequencies of photoperiod non-responsiveness and winter breeding in this species, and to test the effects of season, environmental variables, and body condition on reproductive condition and reproductive timing. There were significant effects of body mass, photoperiod, percent fat, ambient temperature, and snow depth on reproductive organ masses, depending on the sex

and breeding period ($p < 0.05$). One instance of late-summer photoperiod non-responsiveness was observed in a free-living male vole, but no winter breeding. The study showed that up to 28.2% of captive male voles given food *ad libitum* and housed at room temperature were non-responsive to short photoperiods. Both the free-living and captive animals that were non-responsive to photoperiod were in substantially better condition than their photoperiod responsive (*i.e.*, regressed) counterparts, suggesting a link between maintenance of reproductive structures and energy intake. I concluded that photoperiod non-responsive morphs are conserved in at least one arctic/subarctic species at frequencies comparable to lower-latitude voles, but that size and maintenance of reproductive structures in Alaska free-living populations depends heavily on environmental conditions.

Next, I investigated the potential mechanism by which intraspecific variation in reproductive response to short photoperiods occurs in *M. rutilus* (Chapter 4). I measured differences in protein and steroidogenic hormones expressed along the hypothalamic-pituitary-gonad (H-P-G) axis in long day (LD), non-responsive (NR), intermediately responsive (IR), and responsive (R) *M. rutilus* that were identified in my captive trials. My aim was to determine whether phenotypic variation in reproductive response to short photoperiod is regulated at the hypothalamic level by the number of gonadotropin releasing hormone (GnRH) and gonadotropin inhibiting hormone (GnIH) neurons in the hypothalamus, at the pituitary level by the production of luteinizing hormone (LH), or at the gonadal level by the production of testosterone. GnRH was successfully stained in the brains of voles, but GnIH stained only partially. This could have been due to low homology between arvicoline rodent GnIH and quail GnIH (antibody made in quail). Although staining was sufficient to map the position and location of both types of neurons throughout stereotaxic sections of the brain, only staining of GnRH neurons was sufficient for quantifying the number of immunoreactive cells. GnRH cell counts did not differ significantly among groups ($p > 0.05$), but differences still may be occurring in cell area or concentration of hormone

(optical density of GnRH neurons). LH and testosterone showed similar trends in mean values (highest in LD, next highest in NR/IR, and lowest in R), although only testosterone was significantly different between groups. Both LH and testosterone, however, were significantly ($p < 0.05$) correlated with testis mass, and lack of difference between groups for LH may have been due to negative feedback of different hormones on the pituitary. I concluded that intraspecific differences in reproductive response to photoperiod occurs at or above the pituitary level (*i.e.*, differences in production of LH, in GnRH cell area or density, and/or in GnIH).

I next investigated seasonality of energy and mass allocation in free-living *M. rutilus* in southcentral Alaska (Chapter 5). The aim of this study was to determine whether elements of seasonality were related to any of six body composition parameters, any of eight relative visceral internal organ masses, or field metabolic rate (FMR) of northern red-backed voles, as this could convey information about how or why voles undergo a physiological response to season to facilitate either breeding or winter survival. In winter, the percentage protein of voles declined, and mass was diverted away from the spleen and the liver. Body fat percentage did not change seasonally ($p > 0.05$), but relative spleen values increased in summer. Relative cecum and stomach values were highest in spring, and relative length of the small intestine was highest in summer. This was presumably due to increases in food intake or energy requirements associated with reproduction in these seasons. Body condition was most strongly related to photoperiod, while organ masses were strongly tied to gender and ambient temperature. The FMR of voles did not differ between voles (males and females grouped) in breeding and non-breeding seasons ($p = 0.852$) but was correlated with T_a ($R^2 = 0.29$, $p = 0.037$). I concluded that winter is likely to be the most energetically challenging season for voles in this region, and that they allocate mass (and therefore, energy) away from liver, spleen, and total body protein (perhaps muscle) to compensate for increased thermoregulatory demands.

I then began to investigate the seasonality of bone mineral density (BMD) in free-living *M. rutilus* in Alaska using DXA (Chapter 6; Chon *et al.*, 2008; Stevenson *et al.*, 2009a). My aim was to determine whether the BMD of voles changed seasonally, and if so, whether there were effects of body mass or photoperiod on BMD (an indirect measure of activity and sex steroid levels). BMD increased dramatically from the start of spring to a peak level in early summer, and then decreased gradually to their lowest point in late winter. BMD was significantly lower in fall and winter than in spring and early summer. The BMD of long bones was significantly correlated with both body mass and photoperiod, which accounted for 46.2% and 45.7% of the variation in the BMDs of femur and humerus, respectively. I concluded that the seasonal changes in BMD of voles are likely due, in part, to the combined effects of body mass, activity, and baseline levels of reproductive hormones.

Finally, I used DXA to investigate the effect of overwintering on body mass and bone mineral density in two hibernating mammals: the arctic ground squirrel (*Spermophilus parryii*) and the American black bear (*Ursus americanus*) (Chapter 7). These species undergo a 5-7 month period of hibernation in wild populations, during which time little or no mechanical stress is placed on the bones. However, they do not maintain body temperature or metabolic rate during this period as voles do. Therefore, the aim of this study was to measure the effect of hibernation duration and temperature on the body mass and bone density of these animals and to compare this to the effects of overwintering on the nonhibernating rodent, *M. rutilus*, to determine interspecies differences. Femoral bone densities of ground squirrels exhibited no overall change during hibernation ($p > 0.05$), nor did bone densities of the phalynx diaphysis in bears. However, bone densities of diaphysis midpoints decreased slightly in all bears during hibernation (paired t-test, $n = 4$, $p = 0.075$, not significant) and were negatively correlated with date of emergence (simple regression, $p = 0.048$, $R^2 = 0.91$). That BMD did not change in the true hibernator (*S. parryii*), decreased slightly in the weak hibernator (*U. americanus*),

and underwent a pronounced seasonal osteopenia in the non-hibernator (*M. rutilus*) suggests that any winter osteopenia is also reduced. In the case of *S. parryii* it may have been rendered so small that it was undetectable. The ecological significance of these studies is that it is imperative for all three species to enter their respective breeding seasons with strong bone densities in place, whether conserved over hibernation or reduced and rebuilt again in spring.

The diverse phenotypic reproductive response of *M. rutilus* to short day lengths under laboratory conditions suggests that reproductive timing is fairly elastic in this species. Yet, its reproductive and body condition are strongly influenced by acclimatization to season and environmental factors (both photoperiod- and nonphotoperiod-related). The direction, severity, and rate of climate change are likely to determine how this species and its food source will be affected, specifically with regard to temperature and predictability. Depending on air and ocean currents, circumpolar regions could also experience changing levels of rainfall, snow cover, humidity, or wind.

New selective pressures, both direct (through changing biotic factors) and indirect (through modified pressures from competition, predation, parasitism, etc.), are expected to act on northern populations of plants and animals facing arctic warming (Berteaux *et al.* 2004). Changes in climate are likely to have a substantial effect on the reproduction, body composition, and energy allocation of small mammals. Some changes can be predicted for what might occur under the most accepted models predicting climate change, which are generally characterized by a long-term warming trend that could include longer summers, milder winters, and greater unpredictability (reviewed by Walther *et al.*, 2004; Hinzman *et al.*, 2005; see also Cox *et al.*, 2000; Moore *et al.*, 2008). Whether the effect of such a potential trend on winter breeding and winter survival of voles is positive or negative depends on its effect on the thickness of the insulatory winter snow pack and on climactic conditions in spring and fall. If the snowpack is positively affected, vole populations might experience fewer energetic challenges

in winter and increases in frequency of non-responsive morphs and winter breeding events. If snowpack is negatively affected by warmer temperatures (*e.g.*, decreased insulation, less snowfall, or more freeze-thaw events), it could cost voles valuable energy and leave them in poorer condition at the end of winter. This is not ideal for spring and summer breeding. Based on field study results, longer breeding seasons and overall warmer years would leader to higher annual body masses (animals would be larger for longer periods of time). Voles would be likely to have more litters and invest more strongly into mass allocation, which would benefit reproduction at this time provided that warmer winter conditions would allow sufficient overwinter survival to reach summer.

Under the potential scenario of a warming trend of 1-3°C per decade, northern red-backed voles (*M. rutilus*) in summer might be able to breed for longer periods of time or produce more litters per year. If temperature increased over several decades, however, voles would be drawn out of their thermal neutral zone more in summer and less in winter. Assuming no adaptation of voles, this would mean a shift towards a higher thermoregulatory cost in summer and a lower thermoregulatory cost in winter. If this cost began to affect fitness, however, adaptation could manifest itself in differences in pelage or skin thickness, coat color, or body composition to keep up with climate change. Climate-induced adaptation can be rapid in rodents, occuring within a few decades, as described in a detailed analysis of quantative genetic parameters in Yukon Territory red squirrels (*Tamiasciurus hudsonicus*) (Berteaux *et al.* 2004). However, not all species may be able to keep pace with rapid climate change.

In the vole, there is likely to be some selection for maximum fitness leading to population-wide differences in timing of onset and termination of breeding. There might be increased expression of fecundity genes leading to larger litter size (reviewed in McNatty *et al.*, 2004; Fabre *et al.*, 2006), as voles could afford to wean more offspring if food was more readily available and for longer periods of time. The success of predator and secondary prey species that are influenced by

voles might also increase under this scenario. However, the scenario does not take predictability into account. Several years of warming, followed by an unpredictably cold or wet year might cause both arvicoline and predator populations to crash. Thermogenic capacity of voles already decreases during reproduction (Li and Wang, 2005), and such an event could have further negative effects on valuable energy resources and reproduction. Furthermore, there might be less energy available for reproduction in an unseasonably cold year if the immune systems of voles were upregulated in response to declining temperature (Nelson and Demas, 1997; Nelson *et al.*, 1998). Environmental change or different phases of arvicoline rodent population cycles could also impact the physiology of voles by acting on other axes or pathways (*i.e.*, the hypothalamic-pituitary-adrenal axis, the hypothalamic-pituitary-thyroid axis, the central effector pathway; reviewed in Chapter 1).

Presumably, the high arctic can only be inhabited by animals that are extremely photoperiodic (Bronson, 1989). Selection towards populations of voles and lemmings that rely more heavily on non-photoperiodic factors, therefore, might be incompatible with high-arctic living. Populations at these latitudes that cannot use photoperiod as a regular and reliable cue might not be able to maintain viable numbers. However, lemmings in high arctic have been shown to respond reproductively to the emergence of green vegetation in spring (Negus and Berger, 1998), although it is not known how reliable this response is under differeing photoperiods. No reports of green vegetation affecting high-latitude voles have been reported, and in my studies voles always became reproductive prior to emergence of grasses. Circadian rhythm might also be impacted by changes in climate if the home ranges of certain arvicoline rodents shifted northward or Southward. Circadian rhythm is tied to reproduction in many species of rodents, including arvicolines (Bronson, 1989; Nelson, 2000), and a climate-related shift in home range of arvicolines could alter frequencies of circadian rhythm phenotypes within populations.

Up- and downregulation of the H-P-G axis in arvicoline rodents has serious implications with respect to changes in climate and breeding frequency. These animals are entrained to predict annual changes by becoming photorefractory after exposure to a certain number of long days (Wallen and Schneider, 2000). In certain years under climate change scenarios, this could cause early inhibition of reproduction when food was still available (*e.g.,* during an unseasonably warm late summer or fall season). In this case, predators might be able to find and deplete arvicoline rodent populations more easily for longer periods of time.

Conversely, changes in climate could drive selection for higher frequencies of less photoperiodic animals in high-latitude regions (provided that they would be able to survive there). If this occurred, reproductive structures of arvicoline rodents would be regulated at either the pituitary or hypothalamic level via the H-P-G axis and central effector pathway, and they would presumably be in better body condition than their more photoperiodic predecessors (see Chapters 1, 3, 4, 5, and 6). Such animals could partition resources differently in different seasons, further decreasing the intensity by which physiological acclimatization is coupled to photoperiod (see Chapters 3, 4, 5, and 6).

Finally, the physiology and duration of hibernation in certain mammals might also be affected by changes in climate (see Chapter 7). Earlier availability of food might allow these animals to exit hibernacula sooner and could cause them to enter into hibernation later in the year. A change in climate could affect their short, but highly reproductive summers, and might also lead to changes in physiology (*e.g.*, entering or exiting hibernation in better or poorer body condition) or home range (*e.g.,* movement towards areas less susceptible to mid-winter freeze-thaw events or a better winter/summer habitat) as more home ranges become habitable as a result of climate change.

Northern red-backed voles exhibit reproductive flexibility and may breed in winter if environmental conditions enable them, but they are susceptible to effects of season and environmental factors on body composition and energy

expenditure. The identified mechanisms, correlations, and relationships in the above studies improve the current understanding of reproductive regulation and energy allocation in arvicoline rodents. Changes in population cycles, climate, or predictability of food could significantly alter reproduction and resource partitioning in arvicoline rodents and thereby directly affect population numbers of these animals and their predators. I suggest that reproductive fitness may be either positively or negatively affected, depending on which scenario is employed. Further studies on the seasonality of reproduction, body composition, and energy expenditure in arvicoline rodents and other animals will continue to shed new light on the mechanisms underpinning population cycling and response to potential climate change in high-latitude regions.

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